A STUDY OF

~~SCHWANN CELL PRECURSORS AND~~ SCHWANN CELLS: SURVIVAL, PROLIFERATION, ~~MATURATION,~~ AND INTERACTION WITH NEURONS

by

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ABSTRACT

SCHWANN CELL PRECURSORS AND SCHWANN CELLS: SURVIVAL, PROLIFERATION, MATURATION, AND INTERACTION WITH NEURONS

The first part of the studies presented in this thesis shows that NDFβ (but not NDFα), a member of a novel family of growth factors, acts as a long-term survival factor for Schwann cell precursors. The potential of NDFβ to rescue precursors from apoptotic cell death is independent of activation of either IGF receptors or insulin receptors. In contrast, FGF in the presence of IGF only supports precursor survival for 20 hr. The loss of survival activity of this factor in long-term precursor cultures may be due to the fact that precursors lose their responsiveness to FGF. NDFβ not only rescues precursors from apoptotic cell death but also stimulates DNA synthesis in these cells. The mitogenic potential generated by this factor is also independent of both activation of IGF receptors and elevation of cAMP levels. FGF in combination with forskolin, a well known Schwann cell mitogen combination, fails to promote DNA synthesis in Schwann cell precursors. Interestingly, TGFβ acts as a mitogen but not survival factor for these cells. Schwann cell precursors cultured in NDFβ containing medium not only survive, but also develop into Schwann cells as judged by their ability to survive in defined medium and expression of S100. Furthermore, this study also demonstrates that Schwann cell precursors express the NDF receptors: ErbB2 and ErbB4.

The ability of NDF to regulate survival and DNA synthesis in Schwann cell precursors suggests that this molecule may play an important role in the interaction between neurons and precursors. The second part of the thesis shows that both pure neuron conditioned medium and neuronal surface molecules support Schwann cell precursor survival, and neurons, but not Schwann cell precursors, express NDF protein. A soluble ErbB4 protein blocks the survival activity in neuron conditioned medium and that associated with neuronal surfaces, indicating that NDF acts as neuro-glia signalling molecule, mediating precursor survival. Furthermore, the

neuronal signals also induce DNA synthesis in Schwann cell precursors and drive the maturation and differentiation of these cells in the neuron-precursor co-cultures.

The third part of the thesis demonstrates that two different mitogenic assays may give information that can be related to two different proliferation events, i.e. during nerve development and during Wallerian degeneration. In the first assay, using cells which are assayed immediately after dissociation from nerves, FGF, NDF, TGFβ and PDGF have differing mitogenic activities which may reflect the mitogenic activity of these factors in normal nerve. In the second assay, using Schwann cells pre-cultured in serum-containing medium for 5 days, all the factors mentioned above are mitogenic, and the DNA synthesis stimulated by these factors is higher in adult Schwann cells than younger cells, which is in line with the massive proliferation of adult Schwann cells seen in Wallerian degeneration.

CONTENTS

LIST OF ILLUSTRATIVE MATERIAL

ABBREVIATIONS

ARIA; Acetylcholine receptor-inducing activity.
cAMP; Cyclic adenosine monophosphate.
db-cAMP; N6,2'-0-dibutyryladenosine 3':5'-cyclic monophosphate
BDNF; Brain-derived neuronotrophic factor.
BMP; Bone morphogenetic protein.
BrdU; Bromodeoxyuridine.
BSA; Bovine serum albomin.
CNS; Central nervous system.
CNTF; Ciliary neuronotrophic factor.
CREB; cAMP response element binding protein.
CS; Calf serum.
DAG; Diacylglycerol.
DMEM; Dulbecco's modified Eagle's medium.
DRG; Dorsal root ganglia.
E; Embryonic day (eg. E14=embryonic day 14).
EDTA; Ethylenediaminetetraaceticacid.
EGF; Epidermal growth factor.
FCS; Foetal calf serum.
FGF; fibroblast growth factor.
FGF-1; acidic fibroblast growth factor (aFGF).
FGF-2; basic fibroblast growth factor (bFGF).
FGF-3; fibroblast growth factor 3.
FGF-4; Kaposi's fibroblast growth factor (KFGF).
FGF-5; fibroblast growth factor 5.
FGF-6; fibroblast growth factor 6.
FGF-7; Keratinocyte growth factor (KGF).
FGF-8; Androgen-inducible growth factor.
FGF-9; fibroblast growth factor 9.
Gal-C; Galactocerebroside.
GAP-43; Growth associated protein 43kD.
Gas3; Growth arrest specific gene 3.
GFAP; Glial fibrillary acidic protein.
GGF; Glial growth factor.
HEPES; N[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid].
HGF; Hepatocyte growth factor.
HRG; Heregulin.
ICE; Identified protein-interleukin-1β coverting enzyme.
Ig; immunoglobulin.
IGF; Insulin-like growth factor.
LIF; Leukemia inhibitory factor.
MAG; Myelin associated glycoprotein.
MBP; Myelin basic protein.
MEM; Minimal essential medium.
MEM-H; Hepes buffered minimal essential medium.
MIS; Mullerian inhibiting substance.
MIP; Macrophage inflammatory protein.

N-CAM; Neural cell adhesion molecule.
NDF; Neu differentiation factor.
NDGF; Neuron-derived growth factor.
NGF; Nerve growth factor.
p75NGF-R; Low-affinity nerve growth factor receptor.
NT-3; Neuronotrophic factor 3.
NT-4; Neuronotrophic factor 4.
P; Postnatal day (eg. P4= postnatal day 2).
PDGF; Platelet derived growth factor.
PIP2; Phosphatidylinositol bisphosphate.
PKA; Protein kinase A.
PKC; Protein kinase C
PLL; Poly-L-lysine.
PMP22; Peripheral myelin protein, 22kD.
PNS; Peripheral nervous system.
RAN-2; Rat neural antigen-2
SCG; Superior cervical ganglion.
SCIP; Suppressed cAMP-inducible POU.
SDS; Sodium dodecyl sulphate.
SEM; Standard error of mean.
TGFα; Transforming growth factor α.
TGFβ; Transforming growth factor β.
TPA; 12-O-tetradecanoyl phorbol-13-acetate.

CHAPTER 1

GENERAL INTRODUCTION

SCHWANN CELL PRECURSORS AND SCHWANN CELLS: SURVIVAL, PROLIFERATION, MATURATION, AND INTERACTION WITH NEURONS

It has been widely accepted that Schwann cells play an important role in the development and regeneration of the peripheral nervous system (PNS). During early development, neural crest cells give rise to several cell types: these include neuronal precursors , melanoblasts and Schwann cell precursors. The Schwann cell precursor, a intermediate cell in the Schwann cell lineage, undergoes programmed cell death when cultured without neurons in routine medium. In order to support its survival molecules from neurons are necessary. Neuronally derived molecules not only support Schwann cell precursor survival, but also allow them to generate Schwann cells in vitro. In vivo Schwann cells undergo massive proliferation during maturation. After birth, Schwann cells stop dividing and differentiate into two different cell types, myelin-forming and non-myelin-forming Schwann cells (Mirsky and Jessen, 1990; Jessen and Mirsky 1991; Jessen and Mirsky, 1992). Many efforts have been made to explore the molecular basis of the interactions between Schwann cells and neurons that regulate proliferation and differentiation. So far, the molecules derived from neurons that are important for Schwann cell precursor survival, proliferation and maturation have not been identified although FGFs were found to act as survival factor for precursors in short-term culture. Meanwhile, much remains to be learned about the growth factor regulation of Schwann cell proliferation at different developmental stages and under different culture conditions. This study focuses on Schwann cell precursor and Schwann cell survival, proliferation and maturation during development. The interaction between Schwann cells and neurons is explored and molecules from neurons involved in Schwann cell precursors survival are identified

Thesis aims and objectives

1. To explore molecules which cause the survival and proliferation of Schwann cell precursors at early stages of development.

2. To investigate survival, proliferation and maturation of Schwann cell precursors in contact with neurons and also to identify the possible neuronal molecules involved in Schwann cell precursor survival.

3. To study the mitogenic response of Schwann cells derived from rats of different developmental stages in different growth factors and also under different culture conditions.

DEVELOPMENT OF SCHWANN CELLS

At an early embryonic stage, the ectoderm of the embryo thickens in the middle region and forms a neural plate. Soon after this, the lateral edges of this plate are elevated (neural folds) and fuse, finally leading to the formation of the neural tube. Using a fluorescent marker in chicken embryos it has been shown that neural crest cells arise mainly from the dorsal portion of the neural tube (Serbedzija et al., 1989). Trunk neural crest cells undergo widespread migration along characteristic pathways during embyrogenesis, giving rise to neurons, peripheral glia, melanocytes and adrenomedullary cells (Selleck et al., 1993; Stemple and Anderson, 1993; Anderson, 1989; 1993; 1994; Bronner-Fraser, 1994). In the glial lineage, neural crest cells at trunk level meet up with outgrowing axons in the anterior part of each somite, and join with them to form embryonic peripheral nerves and eventually differentiate to form myelin-forming and nonmyelin-forming Schwann cells (Rickman et al., 1985; Loring and Erickson 1987; Le Douarin et al., 1991; 1993; Jessen and Mirsky 1992; Jessen et al., 1994).

Neural crest cells

As mentioned above, neural crest cells are a population of cells that emerge from the dorsal part of the neural tube to migrate to various locations where they finally differentiate into different cell types. It is clear that before they emerge, the neural tube is a single cell layer and contains morphologically similar cells (Selleck et al., 1993; Bronner-Fraser, 1994). By using microinjection of lineage tracers into

individual trunk neural tube cells in birds and mouse, Bronner-Fraser and her colleagues demonstrated that cells arising from the dorsal neural tube were able to form multiple types of neural crest derivatives. Labeled cells were found in the neural tube, dorsal root ganglia (DRG), sympathoadrenal derivatives, Schwann cells and pigment cells (Bronner-Fraser and Fraser, 1988; 1988; Serbedzija et al., 1994). It is unclear why cells in the dorsal region of the neural tube depart from the tube and undergo migration. It has been suggested that disruption of the basement membrane surrounding the neural tube and the loss of high affinity of cell-cell adhesion molecules might allow the escape of the neural crest cells (Akitaya and Bronner-Fraser, 1992; Dupin et al., 1990) In the trunk, neural crest cells migrate along two major pathways: a dorsal migration pathway which gives rise to melanocytes, and a ventral pathway through the somites, involving progenitors which form neurons, adrenomedullary cells and Schwann cells. (Le Douarin, 1982; Le Douarin et al., 1991; Bronner-Fraser, 1993; Bhattacharyya et al., 1994).

It is likely that the final determination of neural crest cell fate occurs progressively during migration and in association with environmental influences. At one time, it was thought that committed precursors of peripheral neurons were present in the neural crest before the migration had started (Ziller et al., 1983). More recently, some chick neural crest cells have been found to express Po protein, a specific marker for the Schwann cell lineage, before they start to migrate along the axons, suggesting, again, early lineage commitment (Bhattacharyya et al., 1991; 1994). On the other hand, evidence for the existence of multipotent stem cells comes from both in vivo and in vitro experiments. In vivo microinjection of dye into a single crest cell shows that in many cases individual cells give rise to several cell types (Bronner-Fraser and Fraser, 1988; 1989; Serbedzija et al., 1994). In vitro, Sieber-Blum and Cohen (1980) first showed that certain clones derived from single neural crest cells in culture contained both catecholaminergic neurons and pigmented cells. Furthermore, clonal culture studies using rat cells show that firstly, single neural crest cells are able to generate homogenous clones containing only neurons or glia and clones that contain both cell types, secondly, multipotent neural crest cells also generate multipotent progeny indicating that neural crest cells are self-renewing multipotent

stem cells (Stemple and Anderson, 1992; Shah et al., 1994; Davis and Temple, 1994). Together, this evidence indicated that variation in at least some molecular signals in the environment may play a role in lineage fate decisions (Bronner-Fraser and Fraser, 1988; Anderson, 1993; 1994; Bronner-Fraser, 1994). Perhaps the clearest evidence of this is a demonstration in which glial growth factor (GGF, also referred to as Neu differentiation factor [NDF], heregulin and ARIA see below) strongly suppresses neuronal differentiation of rat neural crest cells while promoting or allowing glial differentiation (Shah et al., 1994). In the sensory neuron lineage, BDNF may act instructively to direct neural crest cells along the sensory neuron pathway in clonal cultures (Sieber-Blum, 1991), and leukemia inhibitory factor (LIF) has also been shown to promote 50% of cultured neural crest cells to differentiate into neurons (Murphy et al., 1991b; 1992; 1994). In mammals, FGF-1 or FGF-2 was able to stimulate neural crest cells proliferation in both serum, and serum free medium containing neural tube (Bannerman and Pleasure, 1993; Murphy et al., 1994). Furthermore FGF has been shown to promote both avian and rat neural crest cell survival (Heuer et al., 1990; Kalcheim and Neufeld, 1990; Bannerman and Pleasure, 1993). In addition to FGF, NT-3 has also been reported to stimulate proliferation of quail neural crest cells (Kalcheim et al., 1992). A analysis of melanocyte lineage demonstrates that the Steel factor and PDGF may positively affect melanogenesis in the mouse (Nishikawa et al., 1991), and FGF-2 and TGFβ influence neural crest cells commitment to melanogenesis in chick and quail (Stocker et al., 1991; Rogers et al., 1992). Interestingly, FGF-2 and 12-O-tetradecanoyl phorbol-13-acetate (TPA) have been reported to cause Schwann cell precursor transdifferentiation into melanocytes in culture (Hess et al., 1988; Sherman et al 1993). This accumulating evidence further supports the idea that local extracellular signals may play a key role in determining neural crest cell fate.

Schwann Cell Precursors

The Schwann cell precursor has been described as a distinct intermediate cell lying between neural crest cells and Schwann cells (Jessen et al 1991; 1992). A Schwann cell precursor has been isolated from early embryonic peripheral nerve and shows

significant differences in its morphology, antigenic phenotype, survival ability and proliferation from either neural crest cells or Schwann cells. In embryo day (E) 14 rat sciatic nerve, many tight bundles of axons are accompanied by relatively few dividing precursors. These cells contain a small amount of cytoplasm and form relatively long processes to separate the packed axons into several domains (Jessen et al 1994). In vitro, these cells appear more flattened and group-forming in morphology than Schwann cells. They express low affinity nerve growth factor receptor (p75NGF-R) and growth associated protein (GAP-43), but do not express S100 which is frequently used to distinguish Schwann cells from non-glial cells (Jessen et al 1991, Curtis et al., 1992; Stewart et al., 1992). This is in line with early work by Holton and Weston (1982) that indicated that glial cells in early embryonic quail nerves were S100 negative. It has also been shown that early glial cells in human embryonic nerves lack S100 immunoreaction although human Schwann cells express this protein (Kim et al., 1989). In vitro Schwann cell precursors have also been shown to express the cell adhesion molecules N-CAM , L1 and other molecules including the surface antigens A5E3, rat neural antigen-2 (RAN-2) and the intermediate filament protein vimentin. The GAP-43 protein, which is not expressed by neural crest cells, has also been detected in Schwann cell precursors. Interestingly, all these molecules are also expressed by non-myelin-forming Schwann cells in adult. Molecules such as Po protein and 04 lipid antigen are absent in rat Schwann cell precursors cultured for 20 hr (Jessen et al 1994), although recent evidence shows that Po mRNA is detectable in E14 rat peripheral nerve and Schwann cell precursors (Lee M, 1995 personal communication; Zhang et al., 1995). Furthermore, in chicken, Schwann cell precursors and even neural crest cells have been shown to express Po protein (Bhattacharyya A 1991; 1994). Nevertheless, in terms of phenotypic expression, Schwann cell precursors are more like non-myelin-forming Schwann cells than myelin-forming Schwann cells.

In addition to differences in antigenic phenotype and morphology, Schwann cell precursors undergo programmed cell death when deprived of axonal contact and placed in routine serum-containing or serum free media. The death of E14 and E15 Schwann cell precursors in vitro occurs rapidly: although the cells plate readily on the

coverslip and are visible for the first 4-6 hr, nearly all the cells die during the next 10-15 hr (Jessen et al 1994). Under the electron microscope, the death of Schwann cell precursors is accompanied by a typical apoptotic feature, namely a shrunken cell body, condensed chromatin and intact mitochondria (Wyllie et al., 1980). It was found that neuron-conditioned medium had the potential to rescue Schwann cell precursors from apoptosis, whereas glial conditioned medium was ineffective (Jessen et al., 1994). This experiment implies that Schwann cell precursors require neuron-derived molecules for their survival. This raised the question of what was the molecular nature of the neuronal factors which promote Schwann cell precursor survival. A molecule, basic fibroblast growth factor (FGF-2), expressed both in vivo and vitro by neurons and axons (Neuberger and DeVries, 1993; 1993; Weise et al., 1993), was found to support Schwann cell precursor survival in a 20 hr survival assay in the presence of IGF I or II (Jessen et al., 1994; Gavrilovic et al 1995). Other FGF family members including FGF-1 and FGF-4 are all potent survival factors for precursors in combination with IGF using a 20 hr assay. IGF does not rescue precursors in vitro by itself (Gavrilovic et al., 1995). For long term survival, only neuron conditioned medium and FGF-2 in the presence of serum has been shown to enhance precursors survival for at least 4 days (Jessen et al., 1994).

In vivo Schwann cell precursors not only survive but also proliferate (Friede and Samorajski, 1968; Stewart et al., 1993). FGF-2 and FGF-1 have been shown to promote rat and avian neural crest cell proliferation in the presence of serum, neural tube and embryo extract (Bannerman and Pleasure, 1993; Murphy et al 1994). But in serum-free defined medium the combination of FGFs, IGFs and forskolin, a routine mitogen for Schwann cells, fails to induce DNA synthesis of Schwann cell precursors (Gavrilovic et al., 1995). In vivo, it has been shown that following injection of short pulses of brododeoxyuridine (BrdU) into pregnant rats, 16-18% of Schwann cell precursors in E14 embryo have BrdU positive nuclei, indicative of DNA synthesis (Stewart et al., 1993). Therefore, it seems likely that some unidentified neuronal molecules do exist that stimulate Schwann cell precursor proliferation. There are only a few reports that consider Schwann cell precursor maturation. Neuron conditioned medium again has been shown to convert precursors to Schwann cells during a 5 day

culturing period as judged by expression of S-100 and a dramatically increased ability to survive in routine medium (Jessen et al., 1994). The generation of Schwann cells from neural crest cells during relatively long culture periods has been shown by Smith-Thomas and Fawcett in serum and embryo extract media and by Shah et al in GGF plus growth factor cocktail media (Smith-Thomas and Fawcett., 1989; 1990; Shah et al., 1994).

It is well known that neural crest cells are multipotent stem cells which can give rise to several cell types. It is of interest to know whether Schwann cell precursors are committed to the Schwann cell lineage or whether other options are still open for them. Schwann cell precursors from avian embryonic nerves can transdifferentiate into melanocytes by applying FGF-2 or TPA into the culture medium (Ciment et al., 1986; 1990; Kanno et al., 1987; Stocker et al., 1991). Further study shows that TPA can enhance FGF-2 synthesis in Schwann cell precursors and it has been suggested that this endogenous FGF-2 induces transdifferentiation of Schwann cell precursors into melanocytes via an intracrine mechanism (Sherman et al., 1993). TPA has also been shown to decrease protein kinase C (PKC) activities in quail, suggesting that regulation of endogenous levels of PKC plays a role in transformation of Schwann cell precursors of avian into melanocytes (Hess et al., 1988). These studies indicate that rat Schwann cell precursors may still face fate decisions.

Schwann Cells

In vivo Schwann cell precursors will eventually develop into Schwann cells. It has been shown that some cells from E16 nerves start to express calcium binding protein S100, and that the death rate of these cells is lower than that of E14 precursors when these cells are cultured in defined medium for 20 hr. By E17 almost all cells in peripheral nerves express S100, and the survival percentage of cells at this age has reached about 85% (Jessen et al., 1994). Therefore, it is most probable that the conversion of Schwann cell precursors to Schwann cells occurs between E14/E15 and E17/E18 in rat. EM studies of E14 and E17 nerves have, so far, failed to find a great difference in morphology between E14 Schwann cell precursors and E17 Schwann

cells. At E17 cytoplasmic processes of Schwann cells also enclose many axons, but the number of axons enclosed by each Schwann cell appears to be much lower than at E14 although this has not been quantified. The presumptive perineurial cells start to surround nerves around E16 and E17, and Schwann cells at the edge of the nerve appear to be associated with some collagen fibrils. At E18 the first blood vessels can been seen within the nerve. At E 20, isolated Schwann cells surrounding multiple axons can been observed, and the significantly increasing extracellular space and collagen fibrils become predominant in nerve (Hashimoto, Jessen and Mirsky, unpublished data). By birth, the 1:1 relationship between Schwann cells and axons is seen and myelin wraps are detectable in the first 24 hr after birth. The complete maturation of myelin-forming and non-myelin-forming Schwann cells is largely complete 3 weeks later (Ziskind-Conhaim, 1988).

The molecular phenotype of Schwann cells differ from Schwann cell precursors by first expressing S100 at E16. At E17 immature S100 positive Schwann cells start to express the surface lipid protein 04; its function is still unknown. By E18 more than 95% of cells isolated from peripheral nerves are S100 positive and about E18-E19 these cells start to express another molecule, Galactocereboside (Gal-C), and at E20, all S100 Schwann cells express 04, while expression of Gal-C in all Schwann cells only occurs in the 5th postnatal week (Figure 1.1) (Mirsky and Jessen 1990; Mirsky et al 1990: Jessen and Mirsky, 1991). Jessen et al (1985, 1987) suggested that those Schwann cells which expressed Gal-C during the first two-three postnatal weeks would develop into myelin-forming Schwann cells and the Gal-C expression by non-myelin-forming Schwann cells only appeared after that time. Myelin-forming Schwann cells and non-myelin-forming Schwann cells have radically different molecular phenotypes (Table 1.1). Myelin-forming Schwann cells express myelin proteins including Po (which is the major protein of Schwann cell myelin), myelin basic protein (MBP), myelin associated glycoprotein (MAG) and P2 (Mirsky et al., 1980; Trapp et al., 1981; Hahn et al., 1987; Martini and Schachner, 1986). More recently, PMP-22\gas3 has also been shown to be expressed by these cells. Its function may relate to cell growth (SR13) and myelination (CD 25) (Snipes et al., 1992; Bosse et al., 1994; Zoidl et al., 1995). In contrast, the non-myelin-forming

Schwann cells express glial fibrillary acidic protein (GFAP), p75NGF-R, A5E3, Ran-2 (Jessen and Mirsky, 1984; Jessen et al., 1985; 1985; 1987a; 1990), and also the cell surface adhesion molecules N-CAM and L1 (Mirsky et al., 1986; Seiheimer et al., 1989). Both myelin-forming and non-myelin-forming Schwann cells also express some molecules in common. These include S100, Gal-C and 04 as already mentioned (Jessen et al 1985; 1985; Mirsky et al., 1990). More recently, the growth associated protein GAP-43 has also been shown to be expressed selectively by non-myelin-forming Schwann cells (Stewart et al., 1992).

SCHWANN CELL PROLIFERATION

The proliferation of Schwann cells is an important aspect of the development of the PNS (Ratner et al., 1985 ; Mirsky and Jessen, 1990; Eccleston, 1992). During the early developmental stage, Schwann cell precursors at E14 and E15 undergo rapid DNA synthesis. This DNA synthesis increases still further at E16 and peaks at E19. Then with the onset of myelination, Schwann cell proliferation decreases and finally ends after the third postnatal week (Figure 1.2) (Asbury, 1967; Brown and Asbury 1981; Stewart et al., 1993). Thus, Schwann cells or their precursors in vivo undergo a significant proliferation when in contact with axons. While Schwann cells are deprived from axons in neonatal rat the division rate of these cells decreases (Komiyama and Suzuki, 1992). These observations indicate that the proliferation of Schwann cell in vivo may be driven by axon-associated mitogens.

The Schwann cells and neurons co-culture system which is based on culturing DRG explants, has been established for studying the interaction between Schwann cells and neurons (Wood and Bunge 1975). In this co-culture system Schwann cells have been found to proliferate rapidly in contact with neurites, and excision of the neurons results in rapid decrease in the number of dividing cells (Salzer and Bunge, 1980 I). Furthermore, the neurite membrane fraction and axolemma made from brain have also been shown to stimulate Schwann cell proliferation in culture (Salzer et al., 1980 II, Sobue et al., 1984). The neuronal membrane mitogen was later detected on the neurite surface since preventing Schwann cells from direct contact with neurites in

co-culture by using a permeable collagen diaphragm resulted in no proliferation of Schwann cells. (Salzer et al., 1980 III). These experiments indicated that the axon-associated mitogenic effect derived from direct contact with axons although the living axon was not required. In a series of experiments Ratner et al., (1984, 1985, 1988) showed that the neuronal cell surface mitogen for Schwann cells is a heparin-binding protein, that the surface molecule is able to elevate intracellular cAMP levels of Schwann cells, and the mitogenic response to this molecule can be reduced by adding low concentrations of heparin. Furthermore, an anti FGF neutralizing antibody failed to reduce the motigenic function of this molecule which suggests that the mitogenic molecule of the neuronal membrane is not an FGF like heparin-binding protein. The molecular basis of the axonal mitogen was not identified until Morrissey et al., (1995) reported that the rat neuronal surface molecules which stimulated human Schwann cell proliferation could be blocked by anti human Heregulin β 1 (HRGβ1) and anti human ErbB2 antibody in the co-culture system. Heregulin is the human homologue of GGF/NDF and ErbB2 is one of the receptors used by these growth factors (these issues are discussed in more detail later). This results imply that HRG/GGF/NDF is likely to act as a component of the axonal mitogen for Schwann cells.

Before the identification of HRG/GGF/NDF as an axon-associated Schwann cell mitogen many growth factors and agents had been shown to stimulate cultured Schwann cells to proliferate, and the possible mechanisms of proliferation triggered by these factors have also been widely studied. Raff et al (1978) first demonstrated that in purified Schwann cell cultures, cAMP analogues or agents which could elevate intracellular cAMP levels were mitogens for Schwann cells in serum containing medium. A unpurified GGF extracted from bovine pituitary was the first protein found to stimulate the DNA synthesis of Schwann cells (Raff et al 1978; Brockes, 1980). Afterwards, many growth factors have been found to promote Schwann cell proliferation in serum-containing medium, including impure FGF-2, GGF, PDGF and TGFβ (Eccleston et al., 1989; Ridley et al., 1989; Davis and Stroobant, 1990; Hardy et al., 1992). In serum free medium, however, none of these growth factors are mitogens by themselves, except impure GGF (Stewart et al., 1991). Only when these factors were combined with cAMP analogues or agents which elevate intracellular

cAMP level could they act as mitogens in defined serum-free medium (Schubert et al., 1991; Stewart et al., 1991; Rogister et al., 1993). It was found that forskolin, which elevates intracellular cAMP, could significantly increase PDGF receptor mRNA expression in serum-containing medium (Weinmaster and Lemke, 1990). Furthermore IGF receptors, estradiol receptor and ErbB2 mRNA have also been shown to be upregulated by exposure to cAMP analogues or forskolin (Cohen et al., 1992; Schumacher et al., 1993). This evidence suggests that the cooperation of cAMP and growth factors in mitogenesis is likely to result in part from cAMP induction of the corresponding growth factor receptor. In addition to cAMP, other growth factors like IGFs have also been demonstrated to play an important role in Schwann cell proliferation. IGFs act as a mitogen for Schwann cells when synergizing with forskolin in serum-containing medium (Schumacher et al., 1993). In serum free medium, however, addition of FGF-2 or PDGF or TGFβ is necessary for IGF combined with forskolin to promote Schwann cell proliferation (Stewart et al., 1995). This indicates that activating IGF receptors and elevating intracellular cAMP levels are two basic conditions for many growth factors to stimulate Schwann cell proliferation (Schumacher et al., 1993; Stewart et al., 1995). Two exceptions to this general finding exist. The first is GGF/NDF, the other is hepatocyte growth factor (HGF). GGF/NDF alone in defined serum-free medium promotes Schwann cells DNA synthesis although both forskolin and IGFs enhance this effect (Yoshimura et al., 1993; Levi et al., 1995; Stewart et al., 1995). Interestingly, GGF/NDF does not elevate intracellular cAMP levels in a 10 minute assay, but it does upregulate intracellular PKC levels (Yoshimura et al., 1993). GGF/NDF is also able to upregulate its own receptor such as ErbB2 in Schwann cells (Cohen et al., 1992). HGF is a potent mitogen for purified Schwann cells on its own and in this case, simultaneous elevation of cAMP by forskolin suppresses DNA synthesis. Anti-HGF neutralizing antibodies fail to block neuron-stimulated Schwann cell proliferation. Therefore this factor is not the axonal mitogen for Schwann cells (Krasnoselsky et al., 1994).

In addition to above studies more and more proteins continue to be identified as a mitogens for Schwann cells. A novel macrophage inflammatory protein (MIP)1-a has

been shown to stimulate Schwann cell proliferation (Khan and Wigley, 1994). Antibody against the tetraspan protein CD9 also promotes Schwann cell proliferation (Hadjiargyrou and Patterson, 1995). In this study it is unclear whether the antibody is mimicking the action of a putative ligand for CD9 or, alternatively, blocking CD 9 function. These newly discovered Schwann cell mitogens will help us to further understand the molecular interaction between Schwann cells and their environment in vivo. In addition to growth factors and agents that elevate cAMP, extracellular matrix molecules such as laminin, fibronectin and type IV collagen do also, in certain circumstances, stimulate Schwann cell division, and can, therefore, be thought of a third class of Schwann cell mitogens (Eccleston et al., 1987, 1989; Evercooren et al., 1986; McGarvey et al., 1984; Bunge, 1989). Myelin basic protein (MBP) is believed to play an important role in stimulating Schwann cell proliferation during Wallerian degeneration (see below) (Baichwal and DeVries, 1989; Yoshino et al 1987).

Consideration of Schwann cell proliferation leads to a related question which is how Schwann cell withdraws from proliferation to undergo differentiation. In vivo, myelination and proliferation by individual Schwann cells appear to be incompatible. It is still not clear what causes the reduction in Schwann cell proliferation. One possibility is that the proliferation of Schwann cells is suppressed by growth inhibitory factors. It was found that enteric neurons reduced Schwann cell DNA synthesis when Schwann cells were co-cultured with these neurons, and also that short-term cultured Schwann cells secreted some unknown factors which inhibit DNA synthesis in long-term immortalized Schwann cells (Eccleston et al., 1989; 1991). A 55-kDa protein showing strong antiproliferative activity has been isolated from both Schwann cells and Schwannoma conditioned media. (Muir et al., 1990). Furthermore TGFβ-1 and TGFβ-2 have recently been reported to slightly inhibit Schwann cells proliferation in Schwann cells and DRG neurons co-cultures (Guenard et al., 1993; 1995). Alternative possibilities include down-regulation in expression of mitogen receptors or reduction of amount of mitogens in the Schwann cell environment. Nevertheless, withdrawal from proliferation in Schwann cells during nerve development is accompanied by Schwann cell differentiation.

SCHWANN CELL DIFFERENTIATION

The differentiation of Schwann cells into myelin-forming and non-myelin-forming Schwann cells is believed to rely on signals from neurons. Therefore, the identity of neuronal molecules which drive Schwann cell differentiation or instruct Schwann cells to differentiate into two different cell types is of general interest for all neurobiologists.

Many observations support the idea that axonal signals drive Schwann cell differentiation and, in particular, myelination. Thus neither in vivo nor in vitro do Schwann cells form myelin, or express high levels of the myelin components, except when they are in contact with axons or neurites (Bunge et al., 1986; LeBlanc et al., 1987; Trapp et al., 1988). Schwann cells in newborn rat nerves, many of which have just started the myelination program, will abandon myelin synthesis if they are removed from axonal contact. Even myelinated Schwann cells will stop making myelin if they lose contact with axons due to nerve transection in a process called Wallerian degeneration (see below). Myelination can be induced by culturing these cells with neurons in a way which allows close association between neurites and Schwann cells. Such a co-culture system of Schwann cells and neurons has been widely used to study the interaction between Schwann cells and axons. Bunge et al., (1986) demonstrated that myelination occurred in medium containing serum and embryo extract, while in serum-free and extract-free defined medium neurons failed to induce Schwann cells to myelinate. It is probable that under these conditions, they failed to make basement membrane. It was found later that the ascorbic acid in chick embryo extract was necessary for the formation of the basement membrane (Carey and Todd, 1987; Eldridge et al 1987). However, Schwann cells in serum-free medium can be induced to express both elevated levels of Po mRNA and some Po protein when they are co-cultured with neurons although they do not assemble myelin in this system (Morrison et al., 1991; Brunden et al 1990). This suggests that only myelination or myelin assembly by Schwann cells requires basement membrane formation, but not Po induction. In addition to Po, PMP22 another myelin related gene, is also upregulated by contact with axons (Spreyer et al., 1991). But a

somewhat controversial result also indicates that neurons both from chicken and rat are unable to induce Po expression in Schwann cells from chicken in serum free medium (Bhattacharyya et al., 1993). So far the axon-Schwann cell signalling mechanisms which drive Schwann cell differentiation are still unknown in terms of the identity of the neuron associated molecules and Schwann cell receptors. On the other hand , it has been found that an axolemmal membrane preparation can elevate intracellular cAMP levels in cultured Schwann cells (Ratner et al., 1985). This, together with other observations (see below) indicates that elevation of intracellular cAMP levels in Schwann cells may mimic the axonal signals that trigger Schwann cell differentiation.

Sobue and Pleasure (1984) first reported that Gal-C could be re-expressed when cultured neonatal Schwann cells were exposed to cAMP analogues. But no Po induction was seen when short term cultured Schwann cells were treated with cAMP analogues in serum containing medium (Shuman et al., 1988). In contrast, it triggers proliferation of Schwann cells (Sobue et al., 1986). Po and MBP mRNA and also Po protein can only be induced when intracellular cAMP is elevated in long term cultured Schwann cells (Lemke and Chao, 1988). It was unclear why elevation of cAMP levels induced Po in long term cultured Schwann cells but not in short term cultured cells in the presence of serum until Morgan et al, (1991) first demonstrated that myelin-related phenotypes were induced when Schwann cells were exposed to cAMP analogues in absence of serum, indicating that only the cells which do not divide were induced to express high levels of Po. Meanwhile, the cells showing highest levels of Po protein expression also down-regulated GFAP, N-CAM and p75NGF-R in response to cAMP analogues (Morgan et al., 1991; Mews and Meyer, 1993). Further studies indicated that serum, FGF-2 or FGF-1 can negatively regulate the Po gene expression upregulated by cAMP, at least in part by driving cell proliferation (Morgan et al., 1994) which in turn is incompatible with myelin differentiation as discussed above. Thus, only when Schwann cells are withdrawn from serum or FGF or other growth factors which are mitogens for these cells, can Schwann cells be induced to express high levels of Po. TGFβ also suppresses cAMP-induced Po mRNA and protein in Schwann cells and this suppression is not

accompanied by stimulation of DNA synthesis (Mews and Meyer, 1993; Morgan et al., 1994). Together, this evidence suggests that myelin-formation may be driven by a complex mixture of positive and negative regulatory signals during development.

In vivo myelin-forming Schwann cells are always in contact with large axons, whereas non-myelin-forming Schwann cells relate to several small diameter axons. Therefore, two possibilities exist: first the differentiation of Schwann cells may depend directly on axon caliber (Friede et al., 1972); second, differentiation may rely on molecules expressed by different axons (Spencer et al., 1973). Based on an electron microscope study of different cranial nerves in the rat, it was found that onset of myelination by Schwann cells had no direct relationship with axon diameter. This makes it more likely that molecules associated with axons influence Schwann cell myelination (Hahn et al ., 1987). This idea has been further supported by the observation that the expression of Po mRNA and of the myelin-associated transcription factor SCIP (suppressed cAMP inducible POU, also known as Oct 6, Tst-1) in Schwann cells can be up-regulated in a co-culture system in which Schwann cells and neurons have been separated by microporous membrane. This suggests that neurons regulate Schwann cell Po and SCIP genes by diffusible molecules (Bolin and Shooter 1993).

In order to know how these neuronal molecules might influence differentiation paths, it is essential to know that all Schwann cells can be induced into two alternative paths. Aguayo et al (1976) showed that following transplantation of a segment of sympathetic trunk into a transected mouse sciatic nerve, the regenerating axons of the sciatic nerve were able to induce non-myelin-forming Schwann cells derived from the sympathetic trunk to myelinate (Aguayo et al., 1976). Furthermore, when Schwann cells from sympathetic trunk are co-cultured with DRG neurons, both Po mRNA and Po protein are induced (Brunden et al 1990; and Morrison et al 1991), These experiments indicate that non-myelin-forming Schwann cells have not been naturally committed to be a non-myelin-forming Schwann cell, they can be induced to myelinate if appropriate axon-associated signals are provided. This hypothesis has been further supported by co-culturing non-myelin-forming Schwann cells with superior cervical

ganglia (SCG) neurons in defined serum-free medium. It is known that only a few of the axons originating in SCG neurons are normally myelinated, and it was found that the myelin protein Po and its mRNA induction was negligible in this co-culture system (Brunden et al., 1992). The most likely explanation is that the molecular expression by DRG neurons and SCG neurons are different. More recently TGFβ has been shown to block both Schwann cell myelination and the expression of the myelin-related proteins, including Po, MBP and MAG in co-cultures of Schwann cells and DRG neurons (Guenard et al 1995; Einheber et al 1995). It is also found that molecules of non-myelin-forming cells such as p75NGF-R, N-CAM and L1 are expressed in the presence of TGFβ. The expression of GFAP, a molecule only seen on mature non-myelin-forming Schwann cells in vivo, is strikingly increased by 10-fold in the presence of TGFβ in vitro (Mews and Meyer, 1993; Chandross et al., 1995). Furthermore, TGFβ inhibits Schwann cell proliferation by 75% in the co-culture system (Einheber et al., 1995; Guenard et al., 1995). All these observations suggest that TGFβ may serve as an inhibitor of Schwann cell proliferation and myelination and thus, perhaps drive Schwann cells into the non-myelinating pathway. However, other evidence shows that TGFβ not only suppresses Po expression but also represses the expression of Gal-C , 04 and GAP-43 molecules which have been previously reported to be strongly expressed in mature non-myelin-forming Schwann cells (Stewart et al., 1995). Clearly, the picture of TGFβ action on Schwann cells is not simple at present and the neuronal molecules which drive Schwann cells to differentiate into two cell types are still not identified. But the TGFβs will be some of the most interesting molecules to study further in this context. It is also interesting to notice that both Schwann cells and neurons synthesize TGFβ (Einheber et al., 1995; Rogister et al., 1993; Stewart et al., 1995). Interestingly, the interaction of DRG neurons and Schwann cells in vitro results in a reduction of TGFβ synthesis (Scherer et al., 1993; Einheber et al., 1995). In vivo, regenerating axons in sciatic nerve also downregulated the levels of TGFβ mRNA in Schwann cells (Scherer et al., 1993). Therefore, it is possible that downregulation of TGFβ in some axons may result in myelination of these Schwann cells. It would be very interesting to know whether these phenomena are different in the interaction between SCG neurons and Schwann cells.

Although the molecular mechanisms controlling the process of myelination in Schwann cells still remain elusive, some progress has been made in identifying genes related to myelin formation. SCIP, a cAMP-inducible protein, is a member of the Oct transcription factor family. These factors contain a homeobox domain and a DNA binding domain (POU domain). One of the properties shared by this family is their apparent ability to participate in the determination of cell fate. SCIP, as a member of this family, seems to be in some way involved Schwann cell myelination (Monuki et al., 1989; 1993). It was found that SCIP is expressed by myelinating glia of both the CNS and the PNS (Monuki et al., 1989; Suzuki et al., 1990; He et al., 1991). The SCIP expression in Schwann cells were closely associated with axons in developing and regenerating nerves, and neurons in the co-culture system (Scherer et al., 1993; Bolin et al., 1993). The level of SCIP mRNA peaks prior to the peak in myelin gene expression and elevation of intracellular cAMP levels in Schwann cells significantly induces SCIP mRNA expression indicating SCIP might promote myelin gene expression (Monuki et al 1989). But further studies found that SCIP represses the Po promoter in Schwann cells and multiple SCIP binding sites were identified in Po promoter (Monuki et al., 1993). Transgenic mice that express a dominant-negative form of SCIP (ΔSCIP) exhibit overexpression of myelin-specific gene products and hypermyelination, indicating that SCIP might mediate repression of myelination gene (Weinstein et al., 1995). Moreover, expression of SCIP has also been correlated with Schwann cell proliferation (Monuki et al., 1990). Therefore the function of SCIP in Schwann cell differentiation is still controversial. Krox-20, a zinc-finger transcription factor is expressed by neural crest cells, has been reported to be important for Schwann cell myelination (Wilkinson et al., 1989; Topilko et al., 1994). In mouse, Krox-20 is expressed in spinal roots at E12 and more distal peripheral nerves at E15 (Herdegen et al., 1994). In Krox-20 knock out mice myelin proteins such as Po and MBP are absent and myelin is not formed, although the Schwann cell forms a 1:1 relation with large axons. This implies that Krox-20 is likely to control a set of genes required for completion of myelination in the PNS (Topilko et al., 1994).

SCHWANN CELLS IN WALLERIAN DEGENERATION

In the PNS, injury initiates a sequence of characteristic changes in the distal stump of nerves which include: disappearance and fragmentation of axons; myelin sheaths break down; macrophages invade and Schwann cells proliferate. These characteristic changes are referred to as Wallerian degeneration (Fawcett and Keynes, 1990). During Wallerian degeneration Schwann cells undergo de-myelination, change of phenotype expression and proliferation. As discussed previously, Schwann cells in normal peripheral nerves express two distinct phenotypes: myelin phenotypes (Po, MBP, MAG, P2 and PMP-22) and non-myelin phenotypes (GFAP, p75NGF-R, A5E3, Ran-2, N-CAM, L1 and GAP-43) (Mirsky and Jessen, 1990; Jessen and Mirsky, 1991; 1992). In denervated nerve, Schwann cells downregulate myelin molecules such as Po and MBP, and express non-myelin molecules such as N-CAM, L1, p75NGF-R and GAP-43 (Daniloff et al., 1986; Jessen et al., 1987; Martini and Schachner, 1988; Taniuchi et al., 1988; Lemke and Chao, 1988; Curtis et al., 1992). A similar regulation of these molecular phenotypes is also found when Schwann cells are isolated from peripheral nerve and placed in culture although the time course of the reversal of phenotype is earlier (Mirsky, 1990).

Schwann cells in adult peripheral nerve are generally quiescent. When peripheral nerves in adult animals were transected Schwann cells in the distal stump underwent significant proliferation peaking at between 3 and 5 days post-transection (Oaklander and Spencer, 1988; Clemence et al., 1989; Komiyama and Suzuki, 1992). Interestingly, Schwann cells in neonatal sciatic nerve which normally divide at a distinct rate (Komiyama and Suzuki, 1991; Stewart et al., 1993), show a significant decrease of proliferation rate when this nerve is transected (Komiyama and Suzuki, 1992). It was suggested that myelin debris and also myelin associated proteins might contribute to the striking proliferation in adult Schwann cells (Yoshino et al., 1978; Baichwal and DeVries, 1989; DeVries and Baichwal, 1991).

PROGRAMMED CELL DEATH

Programmed cell death is a natural cell death process which is widespread during animal development. It has been known for more than 40 years since Glucksmann (1950) first reported this phenomenon. It is known that cells undergoing programmed cell death die by apoptosis rather than by necrosis (Wyllie et al., 1980; Oppenheim, 1991; Raff, 1992; Williams et al., 1992; Vaux et al., 1994). Cells dying by apoptosis show characteristic morphological changes. In the early stages, the chromatin aggregates into large compact granular masses and later apparently discrete nuclear fragments appear, meanwhile the cytoplasm becomes condensed and microvilli disappear. The whole cell body shrinks but the organelles initially remain intact. Then the dead cells or fragments are rapidly phagocytosed by their neighbors or macrophages (Wyllie et al., 1980). This model of natural cell death is thought to result from activation of a suicide program although the molecular mechanisms are not yet clear (Wyllie et al., 1980; Ellis et al., 1991). Many studies have shown that cells can be rescued from programmed death by the signals from other cells. Levi-Montalcini and her colleagues (1951) first demonstrated that the degree of normal neuronal death during development could be dramatically increased by removing a target in early development. It is clear now that nerve growth factor (NGF) released by their target cells supports the survival of these neurons and rescues them from apoptosis (Barde, 1989). Schwann cell precursors, the cells in E14-E15 peripheral nerve, undergo programmed cell death when they are dissociated from axons in vitro (Jessen et al., 1994). As mentioned previously, protein(s) in neuron conditioned medium or FGFs in combination with IGFs can rescue these cells from the suicide pathway (Jessen et al., 1994; Gavrilovic J et al., 1995). O-2A progenitor cells and oligodendrocytes have also been shown to depend on signals from other cells in vitro, and PDGF, NT-3, IGF, CNTF and LIF are reported to decrease cell death by up to 90% (Barres et al., 1992; 1993; 1994). Results so far suggest that cells during development or some cells in almost all lineages undergo programmed cell death, and the survival chance for each cell may depend on when and how it contacts other cells

to obtain survival signals. This competitive survival principle may efficiently eliminate unwanted cells.

One of the most exciting finding in this field is the identification of specific genes that regulate cell death. Thus, two novel genes ced-3 and ced-4 have been identified as suicide genes in the nematode C elegans (Ellis et al ., 1986; Yuan, 1994). It was found that during embryonic development, 131 of a total 1090 somatic cells died in every organism, and loss-of-function mutations in ced-3 or ced-4 resulted in a blockade of all programmed cell death in the organism (Ellis and Horvitz, 1991). Another gene named ced-9 has been identified as a negative regulator of cell death. Gain-of-function mutations in ced-9 block almost all cell death. Furthermore, loss-of-function mutations in ced-9 result in death of the animal during early development (Hengartner et al., 1992). Mapping and cloning the ced-3 and ced-4 genes indicated that the ced-3 gene encodes a protein of 503 amino acid residues which turns out to be homologous to a previously identified protein-interleukin-1β coverting enzyme (ICE) (Yuan et al., 1993). ICE has also been shown to activate cell death in several cell lines (Miura et al., 1993). More recently another two members of the ICE family have been identified, ICH-1 and Nedd-2. Further study on ICH-1 indicates that there are two isoforms of ICH-1, one is a 435 amino acid protein named ICH-1_L which is homologous to both ICE and ced-3 protein. The other is a truncated version of the ICH-1_L protein called ICH-1_S which contains 312 amino acids. Overexpression of ICH-1_L induces apoptosis, whereas overexpression of ICH-1_S suppresses cell death (Wang et al., 1994). The ced-9 gene encodes a 280 amino acid protein which is homologous to the mammalian proto-oncogene Bcl-2. The Bcl-2 gene was first cloned from the breakpoint of a chromosomal translocation and has been reported to prevent haematopoietic and neuronal cell apoptosis (Garcia et al., 1992; Allsop et al., 1993). Several other Bcl-2 family members have been identified recently including Bcl-Bax, Bax, mcl-1 and A1 (Nunex and Clarke, 1994). Interestingly, heterodimers of Bcl-2/Bax suppress apoptosis while homodimers of Bax promote apoptosis (Miyashita et al., 1994). Further interest in this field is likely to focus on the link between survival growth factors and the regulation of death-promoting or death-suppressing genes.

GROWTH FACTORS AND THEIR RECEPTORS

Since the first discovery of NGF and epidermal growth factor (EGF) in the 1950s (Levi-Montalcini and Hamburger, 1951; Cohen, 1962), peptide growth factors have been recognized as key molecular elements in the regulation of cell survival, proliferation and differentiation both in vivo and vitro. Recently, molecular biology techniques have made it possible to isolate and characterize many growth factors such as FGF, IGF, NDF, PDGF, TGFβ and many others. It is known that many growth factors are unable to pass the hydrophobic cell membrane, and they have to bind to individual cell surface receptors to transduce their signals (Westermark and Heldin, 1989; Raivich and Kreutzberg, 1994; Heldin, 1995). Because of the very important functions that growth factors are likely to have in mediating developmental cell-cell signalling, understanding of the interactions between growth factors, growth factor receptors and signal transduction pathways has become of fundamental importance to developmental biologists.

Neu Differentiation Factors (NDFs)

The search for soluble factors that interacted with the neu (ErbB-2) receptor led to the discovery of a 44 kDa glycoprotein. This protein was able to induce differentiation of human breast cancer cells (MDA-MB231) into mature milk-secreting cells and was therefore named Neu Differentiation Factor (NDF) (Peles et al., 1992). NDF was purified from medium conditioned by ras-transformed fibroblasts. This soon led to a cDNA clone and nucleotide sequence. A recombinant NDF can be produced in transfected COS-7 cells with full biological activity (Wen et al., 1992). Meanwhile several other groups also reported the purification and sequence of proteins of the same family which they named heregulin (HRG) from human breast carcinoma cells (Holmes et al., 1992), glial growth factor (GGF) from bovine pituitary glands (Marchionni et al., 1993) and acetylcholine receptor-inducing activity (ARIA) from chicken brain (Corfas et al., 1993). The primary structural analysis indicates that NDF, HRG, GGF, and ARIA comprise a new family of polypepide factors. All these

proteins turn out to be the products of a single gene, generated by alternative splicing (Marchionni et al., 1993). This gene has been mapped to the short arm of human chromosome 8 (Orr-Urtreger et al., 1993).

Now there are 15 members of the NDF family which have been fully cloned at the molecular level from rodent, human, bovine and avian sources. The structural analysis has indicated that a total of 10 isoforms exist. They are α2a, α2b, α2c, β1a, β2a, β3, β4a, β1, β1-kringle and β1a-ARIA (Table 1.2) (Mudge 1993; Peles and Yarden, 1993; Ben-Baruch and Yarden, 1994). Most isoforms contain seven basic domains: the N-Terminus, a immunoglobulin domain, a spacer domain, an EGF-like domain, a juxtamembrane domain, a transmembrane domain and a cytoplasmic domain (Figure 1.3). The N-terminus is a 50 amino acid long hydrophilic sequence and undergoes proteolytic cleavage upon maturation of the molecule. Since most of the isoforms lack a hydrophobic signal it is thought that the N-terminal sequence combined with the transmembrane domain allows secretion of a pro-molecule. Most isoforms of NDF family share a similar N-terminus except GGFII and ARIA (Holmes et al., 1992; Goodearl et al., 1993; Wen et al., 1994). GGFII has a kringle domain and a hydrophobic 22 amino acid long signal peptide which enables the GGFII molecule to be secreted (Marchionni et al., 1993). The immunoglobulin domain is present in all isoforms. It may promote the dimerization of soluble NDF molecules. The spacer domain is the region that connects the immunoglobulin domain with the EGF-like domain. This 34 amino acid long sequence contains many sites of N- and O-linked glycosylation which may act as attachment sites for heparin sulfate. GGFII and ARIA lack this region (Marchionni et al., 1993; Falls et al., 1993). The EGF-like domain is defined by six cysteine residues that are characteristically spaced over a 40 amino acid sequence, it folds into a typical structure with three disulfide-linked loops, namely a-loop, b-loop and c-loop. It has been demonstrated that this domain acts as the receptor binding site and also as a functional domain of NDF (Nagata et al., 1994; Wen et al., 1994). Two types of EGF-like domain, named α and β isoforms, are found in the 10 isoforms of NDF. They differ in a 19 amino acid sequence in the region of the c-loop (Peles et al., 1993; Wen et al., 1994). The juxtamembrane domain is the most variable region in NDF, and four subtypes of NDF exist which

differ in the amino acid sequence, namely 1, 2, 3 and 4. The transmembrane domain is composed of 23 amino acids. All isoforms of NDF except β3 isoform contain this region. NDFβ3 is not secreted into the medium from transfected COS-7 cells suggesting that this region may also be involved in signalling protein secretion (Wen et al., 1994). The cytoplasmic domain exists in all the transmembrane isoforms of NDFs. It contains a common sequence of 157 amino acids which is called the c-subtype. The a-subtype and b-subtype extend an additional 217 and 39 more amino acids respectively (Figure 1.3).

To gain insight into the biological role of NDFs several groups have examined the expression of NDFs in development in rat, mouse and chicken. In mouse, NDF mRNA signal is found by in situ hybridization in cells of the spinal cord at E9 and the expression of NDF in DRG and ventral horns of the spinal cord is detected at E10. Distinct NDF mRNA positive cells were present in the cerebral cortex and the superficial marginal zone during early development. NDFs are also detected in mesenchymal cells of the lung, intestine, stomach and kidney and in the genital ridge, (Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994). It was also shown that non-neuronal satellite cells and Schwann cells do not express GGF mRNA (Marchionni MA et al., 1993). In rat, NDF mRNA expression is similar to that in mouse, i.e. high expression in motor neurons of the spinal cord and DRG. In brain, most cholinergic neurons are heavily labelled (Wen et al., 1992; 1994; Corfas et al., 1995). An analysis of the distribution of various NDF isoforms indicates that NDFs containing a kringle domain are predominant in the nervous system and that the α isoform is absent in adult mouse brain (Meyer and Birchmeier, 1994). Other studies have also shown that NDFβ1 is a neuronal isoform and NDFα2 is the predominant form in mesenchymal and nonneuronal organs (Wen et al., 1994).

NDFs were originally described as neu ligands but have now been found to bind to a family of tyrosine kinase receptors, including neu\ErbB2\Her-2, ErbB3\Her-3 and ErbB4\Her-4. The neu gene was first described as a mutationally activated proto-oncogene in cell lines derived from neural tumors. It encodes a 185 kDa transmembrane glycoprotein with tyrosine kinase activity termed $p185^{neu}$ (Padhy., et

al 1982; Schechter., et al 1984). The similarity of the neu sequence to that of the epidermal growth factor receptor (EGF-R), originally led to the idea that it acted as a receptor for a unknown growth factor (Bargmamm et al., 1986b). ErbB3\Her-3 was cloned and sequenced on the basis of its homology with ErbB2 and EGF-R by two separate groups. It was found that ErbB3 is a single 6.2 kb specific mRNA encoding a 160 kDa protein. It has all the structural features of a tyrosine kinase receptor, with a single hydrophobic transmembrane domain consisting of 32 amino acids that separates the sequence into a 612-residue extracellular region and a 677-residue cytoplasmic domain. The cytoplasmic domain is strongly homologous with other members of the tyrosine kinase family, and the total protein of ErbB3 has been shown to share 40-50% identity with EGF-R and 40-45% identity with ErbB2 (Kraus et al., 1989; Plowman et al., 1990). More recently a fourth member of the ErbB family has been identified. This is the ErbB4\Her-4 gene which encodes a 180 kDa transmembrane tyrosine kinase. Its extracellular domain is most similar to that of ErbB3, whereas its cytoplasmic kinase domain is 79% and 77% identical with EGF-R and ErbB2 respectively, while it has less identity with ErbB3 (63%) (Plowman et al., 1993).

Although NDF was originally identified as a neu ligand which stimulates the tyrosine phosphorylation of ErbB2 in some human mammary cancer cells, it is known that in certain ovarian cancer cells or in ErbB2 transfected fibroblasts NDF is unable to activate ErbB2 (Peles et al., 1993). Furthermore, Plowman et al.,(1993) demonstrated that NDF was a specific ligand for ErbB4 and it failed to induce phosphorylation of ErbB2 in the absence of ErbB4 (Plowman et al., 1993; Culouscou et al., 1993). NDF also phosphorylates ErbB3 and ErbB2 only when ErbB2 is co-expressed with ErbB3 (Carraway et al., 1994), but when cells are transfected with ErbB3 alone no stimulation of tyrosine phosphorylation is observed (Sliwkowski et al., 1994; Guy et al., 1994). These experiments suggest that extensive heterodimerization of receptors and cross-phosphorylation of heterodimers takes place among the ErbB receptor family, and the lack of intrinsic tyrosine kinase activity in ErbB3 results in no stimulation of phosphorylation in ErbB3 alone (Carraway and Cantley 1994). In a binding study, using a β isoform of NDF it was found that NDFβ bound to ErbB4

with high affinity and to ErbB3 with relatively low affinity; no binding to ErbB2 was detected (Tzahar., et al 1994). This suggests that ErbB4 is the only receptor which on it own can bind NDF and be triggered to phosphorylate. ErbB3 may have a low affinity binding with NDF and carry out no tyrosine phosphorylation by itself, but it may heterodimerize with other receptors, such as ErbB1 and ErbB2 and , in this case, cross-phosphorylate its partner (Soltoff et al., 1994). The lack of binding of NDF to ErbB2 suggests that a unknown ligand may still exist for this receptor (Tzahar et al., 1994; Carraway and Cantley, 1994).

The expression and functional analysis of ErbB receptors has been extensively studied recently. ErbB2 mRNA is expressed at very high levels in the rat sciatic nerves and at lower levels in the brain. During development the levels of ErbB2 mRNA are decreased dramatically in both sciatic nerve and brain (Jin et al., 1993). In cultured Schwann cells and also sciatic nerves the expression both of ErbB2 mRNA and protein is maximal at postnatal days 1 and 7, with substantially lower expression in adulthood (Cohen et al., 1992; Jin et al., 1993). ErbB3 mRNA is detected in neural crest cells and Schwann cell precursors in mouse (Meyer and Birchmeier, 1995). An other study indicated that ErbB3 protein is found at very low levels in peripheral nerve and brain at early developmental stages and to have a high expression in adult nerve and brain (Prigent et al., 1992). In ErbB2 and ErbB4 gene mutated mice, the innervation of cranial neural-crest-derived sensory ganglia, motor neurons and hind brain are markedly affected suggesting that the important function of these receptors in the early development of both PNS and CNS (Lee et al., 1995; Gassmann et al., 1995).

The isolation of the NDF family and their receptors has raised general interest in the function and expression of NDFs in the nervous system. In cultured rat Schwann cells, NDF stimulates DNA synthesis in a dose-dependent manner (Marchionni et al., 1993; Goodearl et al., 1993; Stewart et al., 1995) and some evidence has showed that different isoforms of NDF may differentally affect Schwann cell proliferation and differentiation (Neuberger et al., 1993). In human Schwann cells NDF acts as a effective mitogen (Levi et al., 1995). As discussed earlier, Anderson and his

colleagues recently reported that NDF strongly suppressed neuronal differentiation of rat neural crest stem cells and promoted glial differentiation, suggesting that apart from its function as a mitogen, NDF may act as a diversionary factor to instruct neural crest cells into the Schwann cell lineage or alternatively prevent them from adopting a neuronal fate (Shah et al., 1994). In the CNS, NDF can promote O-2A progenitor cell development into oligodendrocytes, but it has no mitogenic role for this cell type (Vartanian et al., 1994). NDF was also reported to stimulate neurite outgrowth in PC12 cells when the cells were transfected with the ErbB2 gene (Gamett and Cerione, 1994). In developing neuromuscular synapses, NDF may be involved in regulating acetylcholine receptor synthesis via ErbB2 tyrosine phosphorylation (Corfas et al., 1993). In the NDF knock out mouse, Schwann cell precursors and cranial ganglia, but not DRG, fail to develop normally, indicating that NDF may play a role in the fate decisions or survival of these cells (Meyer and Birchmeier, 1995).

Fibroblast Growth Factor (FGF)

Fibroblast growth factor (FGF) was originally identified as an activity in pituitary extracts that stimulated the proliferation of mouse 3T3 cells (Armelin, 1973; Gospodarowicz et al., 1974). The partially purified protein had a 14-16 kDa molecular weight with a basic isoelectric point (Gospodarowicz 1975; 1978a). These pioneer studies leads to the discovery of a large family of growth factors, the FGFs. To date nine members of the FGF family are known. They have been assigned numbers in the order of their identification. Acidic FGF\FGF-1, basic FGF\FGF-2, FGF-3, Kaposi's FGF\FGF-4, FGF-5, FGF-6, keratinocyte growth factor (KGF)\FGF-7, androgen-inducible growth factor\FGF-8 and FGF-9 (Baird, 1994). In this big family, FGF-1 and FGF-2 have been most extensively studied in the last ten years. FGF-2 has four different forms due to variation in the N-terminal (155, 196, 201 and 210 amino acids correspond to 18 kDa, 22 kDa, 22.5 kDa and 24 kDa proteins). The remaining part of FGF-2 shows a high homology to other members of the family including two receptor binding sites (amino acids 32-76 and 114-123), one PKC phosphorylation site (Ser-72) and one protein kinase A phosphorylation site

(Thr-120) (Basilico and Moscatelli, 1992). FGF-1 is a 154 amino acid protein and has a 55% amino acid sequence identity to 18 kDa FGF-2. Interestingly most members of this family have no secretory signal in the N-terminal region except FGF-7 and FGF-9 (Rubin et al., 1989).

FGF genes and proteins have been shown to be expressed in most tissues at a relatively high concentration. In particular FGF-2 mRNA is expressed at much higher levels in brain than in other tissues (Shimasaki et al., 1988). During development, FGF-2 increases significantly from embryonic to postnatal stages in brain (Caday et al., 1990). But recent studies indicate that the 18 kDa FGF-2 mRNA is predominant in embryonic brain and essentially undetectable 20 days after birth, whereas adult rat brain expresses multiple FGF-2 isoforms (Powell et al., 1990; Weise et al., 1993). In the peripheral nervous system both FGF-1 and FGF-2 mRNA and protein have been detected in both embryo and adult rat DRG neurons (Grothe et al., 1991; Neuberger and DeVries 1993), and FGFs are also found . in the cytoplasm of Schwann cells in vitro by immunohistochemistry. In in situ hybridization studies it is shown that the levels of FGF-2 mRNA expression in peripheral nerves is quite constant whereas the levels of its corresponding receptor expression is variable during development (Davis, 1993; Neuberger and DeVries 1993).

Two types of receptors that are relevant for FGF action have been identified: heparan sulfate proteoglycan (HSPGs) which serves as a low affinity receptor that can not transmit a biological response, and several different high-affinity receptors with intrinsic protein tyrosine kinase activity (Basilico and Moscatelli, 1992) . So far four members of the high-affinity FGF receptor family have been isolated and sequenced, namely FGFR-1\Flg\Cek-1, FGFR-2\Bek\Cek-3, FGFR-3\Cek-2 and FGFR-4. FGF-Rs have three immunoglobulin-like loops, a hydrophobic transmembrane region, and a catalytic tyrosine kinase domain. It was found that the third immunoglobulin loop is the important domain for FGF binding (Miki et al., 1991). Several binding affinity studies have indicated that FGFR-1 shows a similar high affinity binding for both FGF-2 and FGF-1, with about a 15-fold lower affinity for FGF-4 (Dionne et al., 1990; Mansukhani et al., 1990). FGFR-2 appears to bind FGF-1, FGF-2 and FGF-4

with similar high affinity, and FGFR-3 and FGFR-4 may have higher affinity binding to FGF-1 than FGF-2. Many studies have demonstrated that heparin-like cell surface molecules are necessary for the induction of a biological activity when FGF binds to its high affinity receptor (Yayon et al., 1991; Ruosishti and Yamaguchi, 1991). Recently it was shown that heparin-like molecules are not required for ligand binding, however, heparin-like molecules induce FGF molecules to oligomerize which results in dimerization of FGF receptors and receptor activation (Spivak-Kroizman et al., 1994).

The expression of FGF receptors in the nervous system has been extensively studied. In the adult rat PNS, FGF-R mRNA is undetectable in contrast, E14-E17 DRG and trigeminal ganglia show distinct neuronal labeling in in situ hybridization experiments. In terms of FGF-R protein, most motorneuron and sensory neurons show weak labelling (Weise et al., 1993). In the developing chicken embryo, three receptor genes: cek-1 (FGFR-1), cek-2 (FGFR-3) and cek-3 (FGFR-2) have been studied. Although all three receptors are expressed in a number of the same tissues, the expression of each receptor within a given tissue is generally specific for different cell types, suggesting that the members of the FGF receptor family may represent cell-type-specific receptors rather than ligand-specific receptors (Patstone et al., 1993).

Biological effects of FGF in the nervous system have been shown both on neurons and glia. As mentioned before, FGF-1, FGF-2 and FGF-4 are survival factors for Schwann cell precursors in vitro if IGF is also provided (Jessen et al., 1994; Gavrilovic et al., 1995). FGFs are also well known mitogens for Schwann cells combined with forskolin and IGF (Davis and Stroobant 1990; Stewart et al., 1992; 1995). In the CNS, FGF-2 has been shown to support survival and neurite outgrowth in several neuronal types (Basilico and Moscatelli, 1992; Barid, 1994). FGF-2 also promotes DNA synthesis in astrocytes and neural precursor cells (Murphy et al., 1990), and can act with other growth factors and cytokines to stimulate NGF synthesis and secretion by astrocytes and fibroblasts (Yoshida et al., 1992; 1992).

Moreover, FGF-2 may also act as transdifferentiation factor to drive Schwann cell precursors into melanocytes in the avian system (Sherman et al., 1993).

Transforming Growth Factor β (TGFβ)

Transforming growth factors were first identified as sarcoma growth factors in the conditioned medium of transformed fibroblasts. Subsequently, two major factors were identified and named TGFα and TGFβ (Hsuan, 1989; Burgess, 1989). It is known that TGFα and TGFβ are two completely different growth factors. TGFβ is widespread in most tissues and in all species, suggesting that this factor may be widely involved in tissue development and repair. TGFβ protein and cDNA was first purified and cloned from human platelets (Assoian et al 1983; Derynck et al 1985). Since then, many family members have been reported. TGFβ-like factors can be subdivided into four families: The TGFβs, the activins/inhibins, Mullerian inhibiting substance (MIS) and decapentaplegic/bone morphogenetic protein (dpp/MBP), comprising more than 28 members in total (Massague, 1990; 1994). In the TGFβ family, 5 members have been shown to have great sequence homology, ranging from 64%-82% (Kondaiah et al 1990). The basic structure of TGFβ includes an N-terminal, pro-region with three N-linked glycosylation sites and c-terminal bioactive domain with 9 cysteines. During secretion, the pro-region is cleaved and combines with the TGFβ bioactive dimer to form a biologically latent complex. This latent complex can be activated in vivo by unknown mechanisms. In vitro it can be activated by exposure to extreme pH (<4 or >9), sodium dodecyl sulfate or plasmin (Lyons et al 1988).

There are three types of TGFβ receptors identified so far, called TGFβ receptor type I, type II and type III, which correspond to 53 kDa, 70-85 kDa and 200-400 kDa molecular weights. It is now clear that TGFβ receptor type I and type II are involved in signalling, whereas the type III, which is a betaglycan, may function as a reservoir or clearance system for bioactive TGFβ (Hsuan 1989; Massague 1990). Cloning of the gene for the type II receptor indicates that this receptor is a transmembrane serine\threonine kinase with a short extracellular region, a transmembrane domain, a

kinase domain and a c-terminal rich in serine and threonine. (Matthews et al 1991). Type I and type II receptors interact as the interdependent component of a heteromeric complex. The type II receptor is required for specific ligand binding and this binding recruits the type I receptor which results in the phosphorylation of serine residues on the type I receptor. This phosphorylation may activate the kinase domain of the type I receptor to transduce the signal to downstream components (Wrana et al., 1992; Yamashita et al 1994; Wrana et al 1994; Heldin, 1995).

TGFβs have been widely detected in the PNS and also in dissociated neurons and Schwann cells (Flanders et al., 1991; Millan et al., 1991; Rogister et al., 1993; Scherer et al., 1993; Stewart et al., 1995). Neurons after lesion in vivo or dissociated in vitro have been shown to secrete a active TGFβ while Schwann cells only release the latent form (Rogister et al 1993; Stewart et al 1995). But other evidence shows that neurons may also secrete latent TGFβ (Einheber et al., 1995). Interestingly regeneration of axons downregulates TGFβ mRNA levels in peripheral nerve (Scherer et al., 1993).

TGFβs have been shown to both inhibit or stimulate proliferation depending on the cell type to which they are applied. In avian neural crest cells, TGFβs promote cell differentiation and melanogenesis (Rogers et al., 1992). In Schwann cells, TGFβs stimulate DNA synthesis in serum containing medium (Eccleston et al., 1989; Ridley et al., 1989; Schubert, 1991; Rogister et al., 1993; Guenard et al 1995), but several papers show that TGFβs like FGFs or PDGF-BB, will not on their own promote Schwann cell proliferation in serum free medium (Stewart et al 1992; Morgan et al., 1994; Mews and Meyer, 1993; Chandross et al 1995). It can, in contrast inhibit proliferation driven by neurons (Guenard et al., 1995;). As discussed previously, TGFβs also downregulate Po, NGF receptor and GFAP in Schwann cells (Mews and Meyer 1993; Morgan et al., 1994; Stewart et al., 1995; Chandross et al., 1995), and upregulate L1 and N-CAM (Einheber et al., 1995; Stewart et al 1995).

Insulin-like Growth Factor (IGF)

The term insulin-like growth factor was proposed due to the fact that the structure of these proteins is highly similar to proinsulin. There are two members in this family in addition to insulin, named IGF-I and IGF-II (Clemmons, 1989; Sara and Hall, 1990). IGF-I and IGF-II have been detected in most mesodermally derived tissues during early development which suggests that IGFs might be involved in embryogenesis (Beck et al 1987). In the nervous system IGF-I has been shown to be expressed at a high levels in fetal brain but its levels decreased significantly postnatally (Sandberg et al 1988). In the oligodendrocyte lineage, IGFs support the survival of both O-2A progenitor cells and oligodendrocytes (Barres et al., 1992). In Schwann cells, IGF-I stimulates cell proliferation in the presence of cAMP analogoues and serum (Schumacher et al., 1993). Both IGF-I and IGF-II have been reported to induce DNA synthesis of Schwann cells in response to FGF-2 and PDGF in serum free medium (Stewart et al 1996). IGFs is also required in combination with FGFs for supporting Schwann cell precursor survival in vitro (Gavrilovic et al., 1995). IGF-I null mutations in mice result in dwarves which die shortly after birth (Liu et al., 1993).

dwarfs / dwarfism

In the early 1970s, IGF was thought to bind exclusively to the insulin receptor. Megyesi et al., (1975) first identified distinct plasma membrane receptors for insulin and IGF which finally led to the isolation and sequencing of two human IGF receptors: type I IGF receptor and type II IGF receptor (Rinderknecht et al., 1978; 1978). Type I receptor is closely related to the insulin receptor. It is a heterotetrameric protein which comprises two α subunits (135 kDa) and two β subunits (90 kDa). Type I receptor was cloned and sequenced in 1986 by Ullrich et al, revealing that the α subunits have a growth factor binding domain and the β subunits contain a tyrosine kinase domain. In contrast to the type I receptor, the type II IGF receptor is monomeric and has no kinase activity (Morgan et al., 1987). Ligand binding studies show that the type I receptor has almost the same affinity for IGF-I and IGF-II but appears to have a very low binding affinity for insulin. In contrast, the type II receptor shows a high affinity for IGF-II and 3-4 fold lower affinity for IGF-I, and no reaction with insulin (Neely et al., 1991; Schofield, 1992). IGF receptors and

the insulin receptor have been shown to be present in all rat and human brain regions including cerebral cortex, olfactory bulb, hippocampus, thalamus, cerebellum and pons (Brownstein and Roth 1981; Gammeltoft et al 1984; 1985; 1988). The amount of the type II receptor is significantly higher in fetal brain than adult brain (Gammeltoft et al., 1988). More recently, mice carrying null mutations of the genes encoding IGF type I or\and IGF-I or IGF-II have been analyzed. It has been shown that between E11 to E12.5, IGF type I receptor is acted on by IGF-II in vivo to induce mitogenic signalling, while from E13.5 onward, IGF type I receptor interacts with both IGF-I and IGF-II (Baker et al., 1993; Liu et al., 1993).

Platelet Derived Growth Factor (PDGF)

PDGF is a hetero- or homodimer consisting of two A and B chains linked by disulphide bridges. So far, three members of this family have been found and named PDGF-AA, PDGF-BB and PDGF-AB (Heldin and Westermark, 1990). In the CNS, PDGF mRNA and protein have been detected in embryonic brain and optic nerve and throughout development until adulthood (Richardson et al., 1988; Pringle et al ., 1989; Yeh et al ., 1991; Sasahara et al., 1991; Reddy and Pleasure, 1992). In culture, type-1 astrocytes have been shown to produce PDGF A chain (Richardson et al., 1988). In vitro, PDGF supports the survival of O-2A progenitor cells and newly formed oligodendrocytes and stimulates the proliferation of O-2A progenitor cells (Noble et al., 1988; Richardson et al., 1988; Barres et al., 1992). In vivo, PDGF decreases normal oligodendrocyte death by up to 90% in developing optic nerve (Barres et al., 1992). PDGF has also been shown to increase Fos and Jun expression in newly formed oligodendrocytes (Hart et al., 1992). In the PNS, PDGF has been detected at relatively high levels in neonatal rat DRG and sciatic nerve, and Schwann cells show a much stronger labeling than neurons. The levels in Schwann cells declined during the first postnatal weeks (Eccleston et al 1990; 1993). It is well known that PDGF in combination with cAMP is a mitogen for Schwann cells (Davis and Stroobant, 1990; Eccleston et al, 1990; Stewart et al., 1992; Hardy et al ., 1992). In view of the interaction between PDGF and IGFs discussed above, it is of

interesting that PDGF increases the levels of IGF receptor mRNA in the c3T3 cell line (Rubini et al., 1994).

The discovery of three PDGF isoforms has led to the identification of two different PDGF receptor types: PDGF α-receptor and β-receptor (Hart et al., 1988; Heldin et al., 1988). It is clear now that the β-receptor binds PDGF-BB with high affinity and PDGF-AB with lower affinity, but does not bind PDGF-AA. Whereas the α-receptor can bind both A and B chains (Heldin and Westermark, 1990). PDGF receptors are widely expressed both in the CNS and PNS. Reddy and Pleasure, (1992) reported that both PDGF α and β receptor mRNA levels are markedly higher in rat brain at E15 and postnatal day 6 than that in later life. It was also found that at E9 PDGF α receptor mRNA was present in the most dorsal portion of the neural tube, associated with early neural crest cells (Schatteman et al., 1992), after E16 the signal is only expressed on glial cells, but not on neurons in the CNS (Pringle et al., 1992). Antisera specific for the PDGF α- and β-receptors revealed high levels of both receptors in the neonatal rat PNS, whereas, both receptors can only be detected in non-myelinated fibres in adult nerve (Eccleston et al., 1993).

Table 1.1 The molecular phenotype of Schwann cells

Antigen	Myelin-forming Schwann cells	Non-myelin-forming Schwann cells	Short-term cultured cells	Precursors
CNPase	+	n.d.	+	n.d.
Po	+	-	-	-
MBP	+	-	-	-
MAG	+	-	-	-
P_{170k}	+	n.d.	-	n.d.
PLP	+	n.d.	+	n.d.
P_2	$+_a$	-	-	n.d
011	+	-	-	n.d.
GFAP	-	+	+	-
p75NGF-R	-	+	+	+
N-CAM	-	+	+	+
L1	$\pm_b$	+	+	+
A5E3	-	+	+	+
Ran-2	-	+	+	+
C461	-	+	n.d.	n.d.
S100	+	+	+	-
Vimentin	+	+	+	+
Laminin	+	+	+	+
Nestin	+	+	+	+
04, 08, 09	+	+	-	-
Gal-C	+	+	-	-
Seminolipid	+	+	+	-
GAP-43	-	+	+	+

[a]Present on some myelin-forming Schwann cells only

[b]Present mainly at nodes of Ranvier

n.d. Not determined.

Adaped from Mirsky R and Jessen KR, (1990): Schwann cell development and regulation of myelination. Seminar in Neuroscience 2:423-435

Table1.2 The different isoforms of NDF family

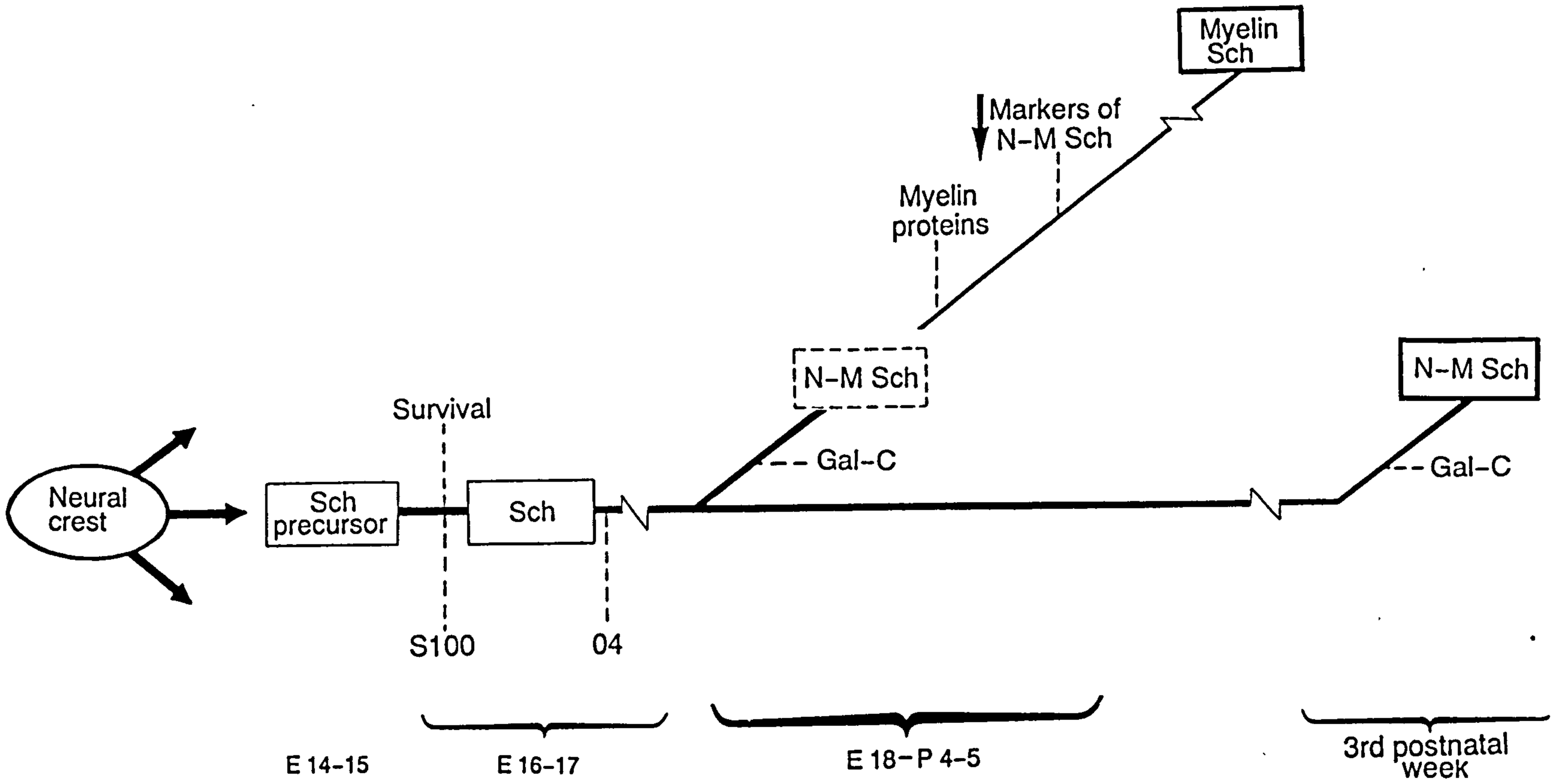
Name	Isoform	Source	Protein	Properties	References
NDF	β1	rat neural tissue	40-44 kDa secreted protein	Mainly in neural tissue	Wen., et al. (1994)
	β2a	Rat1-EJ	40-44 kDa secreted protein 95 kDa proNDF	Mitogenic to Schwann cells	Stewart et al. (1996)
	β3	Rat1-EJ	30 kDa nonglycosylated protein	Not secreted	Wen et al (1994)
	β4a	Rat1-EJ	40-44 kDa secreted protein 95 kDa proNDF	Phosphorylation of Neu in MDA-MB 453 cells	Wen et al (1994)
	α2a	Rat1-EJ	40-44 kDa secreted protein 95 kDa proNDF	Rapind processing	Wen et al (1994)
	α2b	Rat1-EJ	40-44kDa secreted protein 60-75 kDa proNDF	Phosphorylation of New in MDA-MB 453 cells	Wen et al (1994)
	α2c	Rat1-EJ	44 kDa secreted protein 60-75 kDa proNDF	Induces cell growth arrest in 565 breast cancer cells	Wen et al (1994)
Heregulin					
	β1a	MDA-MB-231	45 kDa secreted protein	Mitogenic in SKBR-3 breast cancer cell lines	Holmes et al (1992)
	β2a	MDA-MB-231	-	Phosphorylation of Neu in breast cancer cell lines	Holmes et al (1992)
	β3	MDA-MB-231	-	Not secreted	Holmes et al (1992)
	α2a	MDA-MB-231	45 kDa secreted protein	Mitogneic in SKBR-3 and MCF-7 breast cancer cell lines	Holmes et al (1992)
GGFII	β3-kringle	Human brain spinal cord	45 kDa secreted protein	Mitogenic to Schwann cell	Marchionni et al., (1993)
GGFHFB1	β3	Human brain spinal cord		Not secreted protein	Marchionni et al., (1993)
GGFBPP5	β3	Bovine pituitary cDNA		Not secreted protein	Marchionni et al., (1993)
ARIA	β1a	Chick brain	33-42 kDa secreted protein	Stimulates the synthesis of muscle acetylcholine receptors	Falls et al., 1993

Rat1-EJ: Ras-transformed Rat1 cells.
MDA-MB-231: Human breast cancer cell line.

Modified from Ben-Baruch N and Yarden Y, (1994): Neu differentiation factors: a family of alternatively spliced neuronal and mesenchymal factor. Pro. Soc. Exp. Biol. Med. 206:221-227

Figure 1.1. An outline of Schwann cell development based on the rat sciatic nerve. Sch, Schwann cell. N-M Sch in heavy box, mature non-myelin forming Schwann cells. N-M Sch in stippled box, cells with a molecular phenotype similar to mature non-myelin forming cells that will progress to myelination. Myelin proteins are those proteins including the major myelin protein Po, myelin basic protein, myelin-associated glycoprotein, and others. Markers of non-myelin forming Schwann cells include a GFAP-like intermediate filament protein, N-CAM, L1, and the low affinity nerve growth factor receptor.

Figure 1.1
Development of mature myelin-forming and
non-myelin-forming Schwann cells from neural crest cells



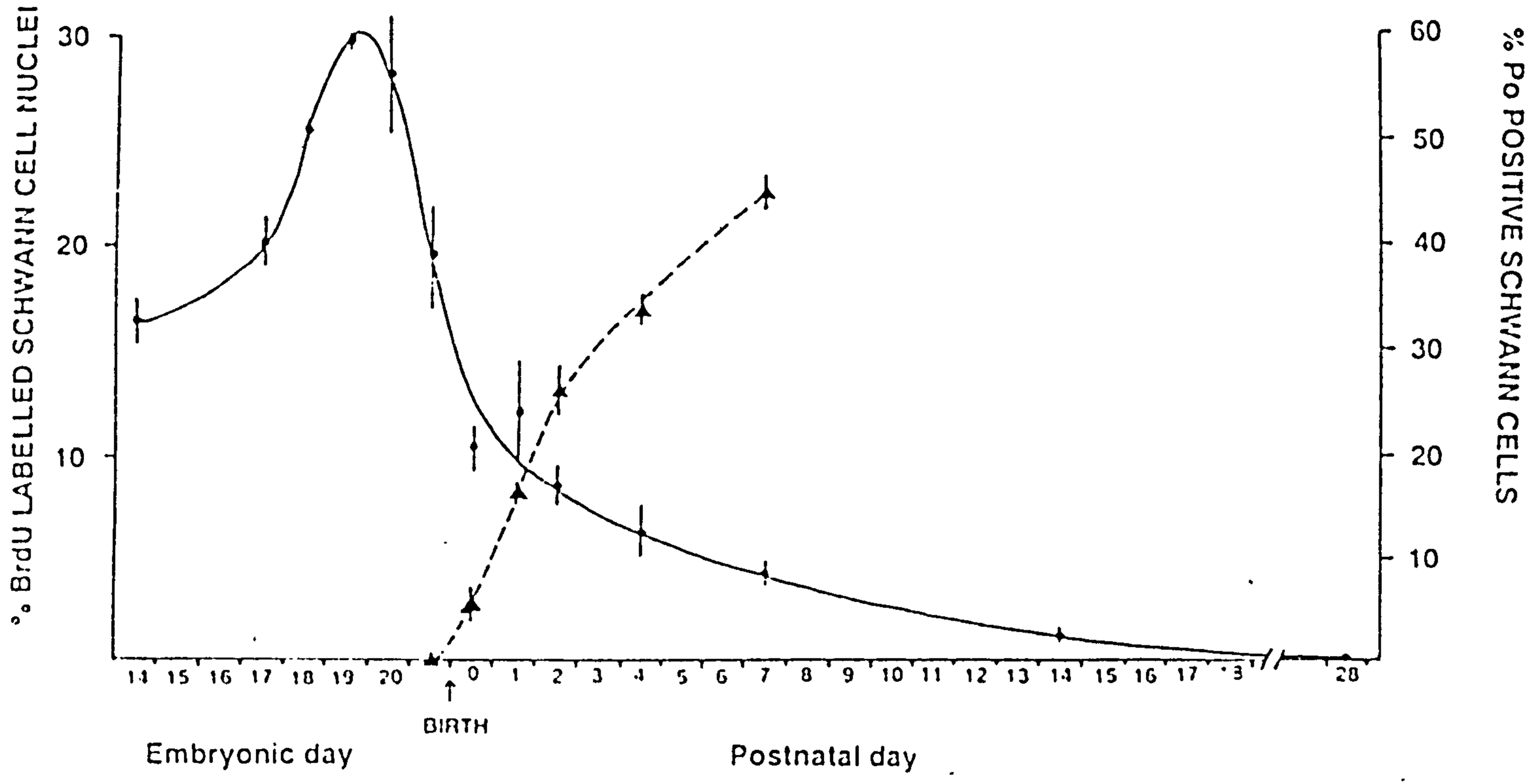
Adapted from Jessen and Mirsky 1992

Jessen, K.R. and Mirsky R. : Schwann cells: early lineage, regulation of porliferation and control of myelin formation. Curr. Opin. Neurobiol. 2:575-581

Figure 1.2. Developmental profile of Schwann cell DNA synthesis in vivo. Schwann cell DNA synthesis was measured by BrdU (1 hr) injection of pregnant and postnatal Sprague-Dawley rats. Sciatic nerves were removed, dissociated and dried down onto gelatin-coated slides, then immunolabelled with BrdU antibodies and 217c (NGF receptor) antibodies to identify Schwann cells (●). Sister perparations from each age were double-immunolabelled with BrdU and Po antibodies (▲).

Figure 1.2

DNA synthesis of Schwann cells during nerve development



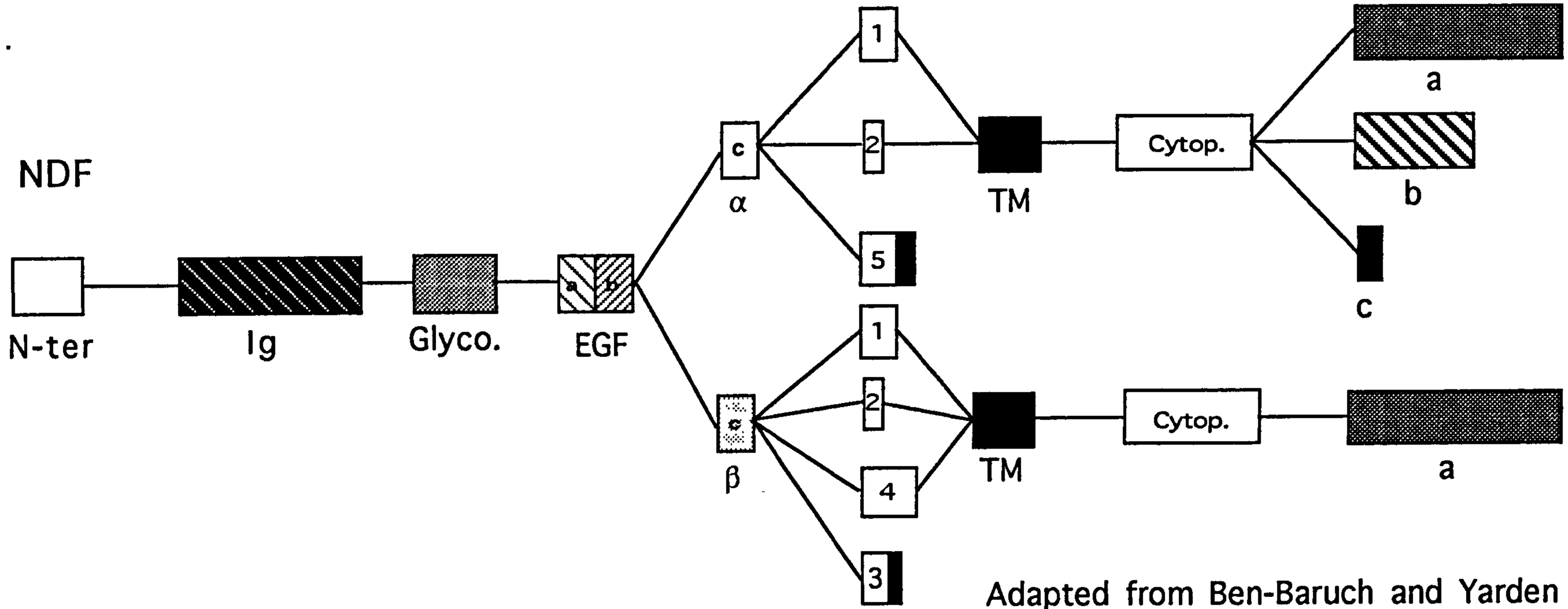
Adapted from Stewart et al., 1993

Stewart HJ, Morgan L, Jessen KR, Mirsky R: Changes in DNA synthesis rate in the Schwann cell lineage in vivo are correlated with the precursor-Schwann cell transition and myelination. Euro. J. Neurosci. 5:1136-1144

Figure 1.3. Schematic representation of the mosaic structure of NDF isoforms and their structural variation. The structure of the two groups of NDFs are represented by boxes that correspond to the major structural motifs. The boxes are drawn to scale and correspond to blocks of coding regions. The recognizable domains include an amino terminal (N-ter) region, an immunoglobulin-like motif (Ig), a glycosylated spacer sequence (Glyco), an EGF-like motif (EGF), which is subdivided into α and β isoforms, a juxtatransmembrane region subdivided into isoforms 1-5, a hydrophobic transmembrane domain (TM), a common cytoplasmic tail (Cytop), and a variable-length cytoplasmic tail (a-c) Note that GGFII contains a putative kringle motif (*Kringle*) and a hydrophobic signal peptide (SP).

Figure 1.3

The Mosaic Structure of NDF/GGF isoforms



Adapted from Ben-Baruch and Yarden (1994): Neu differentiation factor: a family of alternatively spliced neuronal and mesenchymal factors. Proc. Soc. Exp Biol. Med. 206:221-227

CHAPTER 2

MATERIALS AND GENERAL METHODS

Materials and Growth factors

Transferrin, selenium, putrescine, triiodothyronine, thyroxine, dexamethasone, insulin, cytosine arabinoside, hyaluronidase, laminin, poly-L-lysine (Molecular weight 300,000), carbonic anhydrase, N^6,2'-o-dibutyryladenosine 3':5'-cAMP (dbcAMP) and bovine serum albumin (BSA) were obtained from Sigma Chemical Company (Poole, UK). Forskolin was from Calbiochem-Behring Corporation (San Diego, CA). The protein assay kit was from Bio-Rad (Munich, Germany). Tissue culture petri dishes, and 24-well plates were from Falcon (Philip Harris Scientific, London, UK). Four-well plates were from Nunc Life Techno logies (Scotland, UK). NGF was a gift from Dr. J. Winter and brought from Sigma Chemical company (Poole, UK). BDNF and NT-3 were a gift from Drs. G. Dechant and Y. Barde. FGF-1 and FGF-2 was from R & D systems (Abingdon, UK). IGF-1, PDGF-AA, and PDGF-BB were from Pepro Tec Inc. Human recombinant TGFβ-1 was from Boehringer Mannheim (Lewes, UK). Human recombinant EGF was a gift from Dr Fiona Watt. NDFβ-1, NDFβ-2, NDFβ-3, NDF-EGFβ-1, NDF-EGF, NDFα-1 NDFα-2, NDFα-3 and NDF-EGFα-2 were a gift from Amgen Inc (Thousand Oaks, CA.). Calf serum (CS) was from Imperial Laboratories (Andover, UK). Fetal calf serum (FCS) was from Advanced Protein Products (Brierley, UK). DMEM, minimum essential medium, Ham's F12 medium, and trypsin were from GIBCO Laboratories (Paisley, UK). Collagenase was obtained from Worthington Biochemical Corporation (Freehold, NJ). Bromodeoxyuridine (BrdU) was from Boehringer Mannheim (Lewes, UK). Citifluor was obtained from City University (London, UK). XAR-2 and X-Omat film were from Kodak (Rochester, NY). 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium salt were from Boehringer Mannheim (Lewes, UK). All electrophoresis apparatus and reagents were from Bio Rad (Munich, Germany). Nitrocellulose paper was from Schleicher and Schuell (Dassel, Germany). 5(and-6) carboxyfluorescein diacetate, succinimidyl ester (CFSE) was obtained from Molecular Probes Inc (Eugene, OR).

Antibodies

Rabbit antiserum to bovine S-100 protein from Dakopatts (Copenhagen, Denmark) was used at a dilution of 1:10000. Monoclonal antibody 192 IgG1, which recognizes the low affinity p75 nerve growth factor receptor (p75NGF-R) was a gift from E. Johnson Jr. and used in the form of ascites fluid, at a dilution of 1:100. Monoclonal antibody 217c IgG2a was a gift from Dr K. Fields, and used at a dilution of 1:500. Monoclonal anti neurofilament antibody (RT97) was a gift from Dr. J Wood (Anderton et al., 1982). Monoclonal antibody against BrdU was a gift from M. Jones and Dr. K. Mason and used at a dilution of 1:100. Two different rabbit polyclonal anti Po antibodies were used. One was a gift from Dr. B.D. Trapp and was used in diluted 1:500, and the other one was made in this laboratory essentially as described by Brockes et al., (1980) and used in dilution of 1:8,000. Affinity purified rabbit antiserum 1915[#] to recombinant human NDF was used at a concentration of 0.1-10μg/ml for blocking and at a concentration of 5-10μg/ml for immunostaining, rabbit antiserum to recombinant rat NDF was used at a concentration of 1:50, monoclonal antibody 5D6A against recmbinant NDF was used at a concentration of 5-10μg/ml, monoclonal antibody 114A specifically against recombinant NDFβ isoforms was used at a concentration of 0.1-10μg/ml, monoclonal antibody 1H7A4 against recombinant NDFα isoforms was used at concentration of 1-10μg/ml. All monoclonal and polyclonal anti NDF antibodies were from Amgen Inc (Thousand Oaks, CA). Polyclonal antibody K-15 to ErbB-2 receptor and polyclonal antibody C-18 to ErbB-4 receptor were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Both of them were used at a concentration of 1μg/ml. Fluorescein conjugated to goat anti-rabbit immunoglobulin from Cappel Labs. Inc (Durham, NC), absorbed with mouse immunoglobulin to remove cross-reacting antibodies, and tetramethyl rhodamine conjugated to goat antimouse immunoglobulin from Cappel Labs. Inc (Durham, NC), absorbed with rabbit immunoglobulin to remove cross-reacting antibodies, were both used at a dilution of 1:100. Biotinylated sheep anti-rabbit and anti-mouse immunnoglobulins and streptavidin-fluorescein were from Amersham International (Bucks, UK). Polyclonal sheep anti-digoxygenin conjugated to alkaline phosphatase

was from Boehringer Mannheim (Lewes, UK). The ECL chemiluminescence kit and the species specific anti-rabbit Ig conjugated to horse radish peroxidase were from Amersham International (Bucks, UK). The hybridoma cell lines ASCS4 secreting antibodies to the cell adhesion molecule L1 (Sweadner, 1983), was a gift from Dr. A. Furley, the hybridoma cell line OX7 secreting antibodies agaist to Thy 1.1 was obtained from European Collection of Animal Cell Cultures (Salisbury UK), the hybridoma cell line secreting antibodies to the 04 antigen was a gifts from Dr. I. Sommer (Sommer and Schachner, 1981). All these secreting antibodies were used in the form of cell culture supernatant. Vectastain™ Elite ABC kit was from Vector Laboratories (Peterborough, UK).

Defined medium

Defined medium used in this study was a modification of the medium of Bottenstein and Sato (1979). A 1:1 mixture of DMEM and Ham's F12 was supplemented with (final concentration in parentheses) transferrin (100 μg/ml), progesterone (60ng/ml), putrescine (16μg/ml), insulin (10^{-9} M), thyroxine (0.4μg/ml), selenium (160 ng/ml), triiodothyronine (10.1 ng/ml), dexamethasone(38 ng/ml), glucose (7.9mg/ml), bovine serum albumin (0.3mg/ml), penicillin (100IU/ml), streptomycin (100IU/ml), and glutamine (2 mM). In some experiments insulin was used at high concentration (10^{-6} M) or omitted from the medium; this is indicated in the text.

Coating of coverslips and wells

Glass coverslips (13mm diameter) were sterilized and then incubated with poly-L-lysine (PLL) (1 mg/ml) at room temperature for 24 hr, washed with 12 changes of sterile distilled water over a period of 4 days, and allowed to dry. Laminin-coated coverslips were prepared by incubating PLL coated coverslips with 10-30 μl of laminin solution (20μg/ml in DMEM) for 1 hr before plating with cells. Plastic tissue culture wells were coated with PLL by incubating them with 20 μg/ml of PLL for 2 hr at room temperation, draining and air drying.

Preparation of Schwann cell precursor cultures

Cultures of Schwann cell precursors were prepared essentially as described previously (Jessen et al., 1990). Briefly, pregnant rats were killed by exposure to CO_2, and the embryos aged E14 or E13 were decapitated and kept in L-15 medium on ice. To date the embryos, the day of the vaginal plug was counted as E0 (Christie 1965). The sciatic nerves and brachial plexus nerves were dissected out, dissociated, chopped and incubated in enzyme solution containing collagenase (2mg/ml), hyaluronidase (1.2mg/ml) and trypsin inhibitor (0.3mg/ml) for 1-1.5 h at 37°C. The cells were then washed in 6-8ml defined medium. After centrifugation, the cells were resuspended in low insulin defined medium without serum, counted, and plated at a density of 2000 cells per coverslip in a 10 µl drop on laminin-coated coverslips. After 2.5 hr, the cultures were topped up with 380 µl of different experimental media.

Preparation of freshly dissociated Schwann cells

The sciatic nerves from E16 to new born rats, and also from adult sympthetic trunk nerves were dissected and digested in enzyme cocktail as described above for 1.5-2.5 hr according to embryonic and postanatal ages. After washing and centrifugation (as above), 2000 cells were then plated on laminin coated coverslips in 10 µl of defined medium. The cells were topped up to 400 µl of different culture media after 2.5-4 hr according to age. The adult freshly dissociated Schwann cells from sciatic nerves were obtained in the way described below.

Preparation of Schwann cells for purification

Sciatic nerves were dissected from postnatal rats aged newborn, 4 days and adult (above 4 weeks) and desheathed. The tissues were dissociated by a modified method of Brockes et al. (1979). Briefly, newborn and 4 day rat sciatic nerves were dissociated in 0.2% collagenase and 0.125% trypsin in calcium and magnesium-free DME at 37^0 C and 5% CO_2 /95% air for 35 min. Adult sciatic nerves were dissociated in 0.2% collagenase and 0.25% trypsin for 1.5 hr, the same enzymes were then added for another 1-1.5 hr. After incubation a equal volume of MEM-HEPES containing 10% fetal calf serum or calf serum was added and the tissue gently

disoociated. The cells were centrifuged at 500 x g for 10 minutes, resuspeneded in low insulin or high insulin defined medium. The dissociated Schwann cells were cultured in a flask ($25cm^2$) for further purification. In some cases, the cells were plated on coverslips immediately to test the effect of culture period and pre-exposure to serum.

Schwann cell purification

Schwann cells prepared by the above method were cultured in a $25cm^2$ flask for 4 days with Dulbecco's modified Eagles' medium (DMEM) containing 10% fetal calf serum supplemented with penicillin (100IU/ml), streptomycin (100μg/ml), and glutamine (2mM). Cytosine arabinoside (10^{-5} M) was added after 24 hr for 72 hr to kill rapidly dividing fibroblasts. On the fourth day, the cells were detached from the flask with calcium- and magnesium- free MEM-HEPES containing 0.05% trypsin and 0.2% EDTA for 2-3 minutes at 37^0 C for newborn and 4 day rat Schwann cells, and containing 0.075% trypsin and 0.2% EDTA for 4-5 minutes for adult Schwann cells. This procedure could further purify the Schwann cells since the Schwann cells detached from flask before the fibroblasts due the the cellular area difference between Schwann cells and fibroblasts. Detached cells were pelleted by centrifugation at 500 X G for 10 minutes. The purified Schwann cells were then resuspended in defined medium with low or high insulin and plated on laminin coated coverslips at a density of 10,000 cells /coverslip in a 30 μl drop.

Schwann cell precursor survival assay

Schwann cell precursors were plated on coverslips and topped up with different experimental media after 2.5 hr as described above. Then the precursor cultures were fixed by using 2% paraformaldehyde at 3 hr, 20 hr, 44 hr and 68 hr respectively. After immuno-labelling with L1 antibody (see below), the number of the cells counted at the 3 hr point was taken as the total number of Schwann cell precursors alive at the beginning of the assay. This is based on the observation that almost all cells plated on laminin coated coverslips would attach to coverslips during the first 2.5-3 hr. The total number of cells counted at 20 hr or 44 hr and 68 hr were

then divided by the total number of cells at 3 hr, to give the survival percentage at respective time points.

DNA synthesis assay

Two different DNA synthesis assays have been used in this thesis. For the 20 hr assay of freshly dissociated Schwann cell precursors and Schwann cells, DNA synthesis was monitored by exposing cells to BrdU (20μM) for 1.5 hr pulse at 18.5 hr after plating. In some experiments, the cultured cells were exposed to BrdU three times at 3.5 hr, 13.5 hr and 18.5 hr respectively for 1.5 hr pulses. For purified Schwann cells (pre-serum exposed), BrdU (10μM) was introduced for the last 24 hr of a 48 hr assay. The cells from both types of assay were followed by double labelling with L1 and BrdU antibodies for precursors and embyronic Schwann cells, or a p75NGF-R (or S100) and BrdU double label in most Schwann cell experiments (see below). Some experiments were triple labelled with L1, p75NGF-R and BrdU. The percentage of BrdU positive cells among the L1 positive cells was taken as the proportion of Schwann cell precursors synthesizing DNA, and the percentage of BrdU positive cells among the p75NGF-R positive cells was taken as proportion of Schwann cells synthesizing DNA. BrdU labelling was carried out according to Gratzner, 1982. The cells, after labeling with L1 or p75NGF-R, were washed and fixed in methanol (-20^{0}C) for 10 minutes followed by 2 M HCl for 20 minutes to denature DNA. After another washing, cells were incubated with 0.1 M sodium borate for 10 minutes to neutralize the acid, followed by incubation with BrdU antibodies in PBS containing 0.1% Triton X-100 for 45 minutes. After washing the cells were incubated with rhodamine conjugated goat anti-mouse immunoglobulins (1:100) for 30 minutes, washed and mounted in Citifluor anti-fade mounting medium.

Preparation of neuronal cultures from dorsal root ganglia

DRG were dissected from newborn, E15 and E14 rats. 35-40 ganglia were obtained from each rat and incubated with 1.25mg/ml trypsin for 7-15 minutes at room temperature according to the age of rat from which the ganglia were derived. Following addition of 5-6 ml of DMEM or defined medium containing 10% fetal

calf serum, the dissociated cells in suspension were drawn through needles of decreasing diameter: 0.8 mm, 0.6 mm, and 0.5 mm (3-4 times through each needle). The resulting cell suspension was centrifuged at 500 xg for 10 minutes, and the enzyme solution was removed. The cells were either resuspended in low insulin defined medium containing 50ng/ml NGF for culture, or resuspended in DMEM plus 10% fetal calf serum and NGF for further purification.

Purification of DRG neurons by an immunopanning method

Dissociated DRG neurons were resuspended in 6 ml of DMEM containing 10% fetal calf serum and 1-5ng/ml NGF as described above. The suspension was then transferred to a 90 mm plastic non-culture dish coated with anti Thy 1.1 antibodies, since Thy-1 is known to be expressed on both neurons and fibroblasts, but not by glial cells (Fields et al., 1978). After 2-3 minutes at 37^0 C the dish was rinsed very quickly with DMEM or defined medium 4-5 times. The pure neurons attached to the bottom of dish were then streamed off with 10 ml of defined medium containing 5-50ng/ml NGF several times. After centrifugation, the pellet of pure neurons was resuspended in low insulin defined medium containing 50ng/ml NGF and 50ng/ml IGF-1. The neuron suspension was then plated and cultured on coverslips for 20 hr, and labeled with anti neurofilament antibodies (RT97) for neurons and S100 antibodies for glia. All the neurons and non-neuronal cells were counted, and the purity of neurons generated from this immunopanning method was 95%±5.3.

To prepare the anti Thy 1.1 antibody coated dish, the 90 mm^2 non-culture dish was incubated with 6-7 ml of Tris buffer solution (50 mM pH 9.5) containing 50-60μg/ml (40μl) of rabbit anti mouse immunoglobulin overnight at 4^0 C, then the dish was washed 3 times with PBS and further incubated with a anti Thy 1.1 antibodies (the antibodies were in the form of culture supernatant from the OX-7 cell line, and could be diluted in MEM-HEPES according to the concentration of antibodies in supernatant) for at least 1 hr at room temperature. The antibody coated dish was washed 3 times with PBS and used immediately..

Neuron conditioned medium (NCM)

To collect the NCM, 100,000-150,000 neurons purified from DRG of 3 rats were plated in 4-well poly-L-lysine coated plastic plates in 300-400µl of low insulin defined medium containing 50ng/ml of NGF and 50ng/ml of IGF-1. At 20 hr, the medium was replaced with 500-600µl of fresh medium, and cultured for another 48 hr. The NCM was collected at 72 hr after plating. The NCM could either be used immediately or stored frozen (-70^0 C) in cryotubes coated with heat-treated bovine serum albumin.

Schwann cell precursor and neuron co-cultures

For co-culture the neurons were pre-labelled with the fluorecent dye, 5(and 6) carboxyfluorescein diacetate, succinimidyl ester (CFSE). The CFSE (30µM) was introduced into the neuronal suspension for 30 minutes at 37^0 C just before the immunopanning procedure. After labelling with CFSE the neuronal suspension was purified by the immunopanning method described above. Therefore, the CFSE in suspension was washed out during this procedure. For the survival assay, 250 neurons in 60 µl of low insulin defined medium containing NGF (50ng/ml) were plated onto each laminin coated coverslip. The sparse neurons were cultured for 20 hr to allow neurite outgrowth, and during this period the medium could be topped up to 100µl or changed once. 2000 Schwann cell precursors prepared as described earlier were added to the cultured neurons in 60 µl of fresh low insulin defined medium containing NGF (50ng/ml). 340 µl of the same medium was added after 2.5-3 hr. The co-culture was ended at 3hr and 20hr. After L1 immuno-labelling the total numbers of precursors in contact with neurons or neurites was counted and the total number of precursors not in contact with neurons or neurites was also counted. The percentage of survival at 20 hr represents the total number of cells at 20 hr divided by the total number of cells at 3 hr. In this co-culture all the original neurons or non-neuronal cells could be easily distingushed from Schwann cell precursors since the CFSE is fluorescent and is present in neuronal cell bodies, neurites and original Schwann cells. For the proliferation and differentiation assay 2000 CFSE labeled and purified neurons were plated on each coverslip and 4000 Schwann cell precursors were added to the

neuronal culture on the second day. The neuron and Schwann cell precursor co-culture was kept for 1 day, 2 days, 4 days, 6 days, 8 days or 2 weeks for proliferation assays, and cultured for 1 day, 2 days, 4 days, 1 week or 2 weeks for maturation and differentiation assays. Half the medium was replaced each day. A 20 hr BrdU pulse was introduced to the co-culture at each time point for the proliferation assay, and S100, 04 and Po immunolabelling were used for maturation and differentiation assays (for details see immunocytochemistry procedures).

Blocking experiments in NCM or the neuron-precursor co-culture system

To block the survival activity of the NCM, the soluble ErbB4 proteins and DG-2 antibodies were mixed with NCM for at least 2 hr at 37^0C before NCM was applied to the precursors. Then the mixture of NCM and proteins or antibodies was added at 3 hr to cultured Schwann cell precursors. To block the neuron and Schwann cell precursor co-cultures, the soluble ErbB4 proteins were introduced into culture 3 times. First the soluble ErbB4 proteins were added to the neuronal culture at 3-4 hr before the Schwann cell precursors were plated on neurons, secondly, the soluble ErbB4 proteins were mixed with Schwann cell precursors to be plated on neurons, thirdly, the soluble ErbB4 proteins were introduced into the co-culture 5 hr after Schwann cell precursors were plated. Control experiments were carried out by incubating the soluble ErbB4 proteins or DG-2 antibodies with NDFβ-2 and FGF-2 containing medium for at least 2 hr prior to addition to Schwann cell precursors in the 20 hr survival assay.

Immunocytochemistry

To immunolabel cells for the p75NGF-R receptor and L1, cells were fixed in 2% paraformaldehyde for 5 minute, rinsed and then incubated with monoclonal antibody 192 IgG to p75NGF-R or monoclonal antibody ASCS4 to L1 for 30-60 minutes. After washing in minimum essential medium cells were then incubated with biotinylated anti-mouse IgG for 30 minutes followed by streptavidin-fluorescein for 15 minutes. For p75NGF-R and L1 double labelling monoclonal antibody 217c IgG2a and ASCS4 IgG1 were used, and then Texas red anti-mouse IgG2a and biotinylated

anti-mouse IgG1 were applied as the second layers. For staining these cells, antibodies (192 IgG1, 217c IgG2a, ASCS4 IgG1) were diluted in minimum essential medium containing 15 mM HEPES buffer plus 10% calf serum.

For S-100 cells were fixed in 4% paraformaldehyde for 20 minutes, blocked in 3% gelatin PBS for 1 hr, then incubated with anti S-100 antibody (in PBS plus 10% calf serum, 0.02% sodium azide, 0.1 M lysine medium) 1 hr, followed by fluorescein labelling. For 04 labelling co-cultured cells were fixed in 4% paraformaldehyde for 10 minutes, followed by a 1:1 dilution of supernatant containing mouse 04 antibodies for 1 hr, followed by goat anti-mouse Ig fluorescein. For Po labelling, co-cultured cells were fixed in 2 M HCl at room temperature for 15 minutes, followed by 10 minutes in 0.1 M sodium borate. After washing in PBS, the cells were incubated for 1-2 hr in antibody diluting solution (PBS containing 10% FCS, 0.1 M lysine and 0.02% sodium azide). Cells were then incubated in Po antibody (1:8000) in the same solution overnight. The cells were subsequently washed and incubated in donkey anti-rabbit Ig biotin for 1 hr, followed by streptavidin-fluorescein for 15 minutes.

For ErbB2 and ErbB4, cells were fixed in 4% paraformaldehyde for at least 20 minutes, incubated in 0.1% Triton X100 for 10 minutes followed by anti ErbB2 and ErbB4 antibodies for 1 hr, then followed by biotin-streptavidin as above. For immunohistochemistry controls, omission of the first antibody layer was used in every case as a standard control. For ErbB receptor staining, experiments were carried out in which ErbB2 was pre-absorbed with both ErbB2 and ErbB4 control peptides, and ErbB4 was pre-absorbed with both ErbB4 and ErbB2 control peptides for at least 2 hr before staining. This process further comfirmed the antibody specificity and excluded non-specific absorption.

For NDF labelling, neurons or Schwann cell precursors were fixed in 2% paraformaldehyde for 5-7 minutes, followed by anti NDF antibodies (1915# polyclonal anti NDF antibody 1-10μg/ml, 5D6A monoclonal anti NDF antibody 1-10μg/ml, 1H7A4 monoclonal anti NDFα antibody 1-10μg/ml and 114A monoclonal

anti NDFβ antibody 10-50μg/ml) in MEM-HEPES containing 10% calf serum for 1 hr. The donkey anti rabbit Ig biotin or rabbit anti-mouse Ig biotin were applied for 30 minutes, followed by streptavidin-fluorescein for 15 minutes. Control experiments for NDF labelling were carried out by absorbing 5D6A or 1915# antibodies with NDF for 2 hr prior to labelling.

In situ hybridisation

A digoxigenin-labelled cDNA probe was used to detect Po mRNA in Schwann cell precursor and neuron co-cultures, detecting hydridisation by alkaline phosphatase-liked immunohistochemistry. A cDNA (SN63c) encoding the entire Po coding sequence (1.8kb) subcloned into pGEM4, donated by Drs G. Lemke and I. Griffiths was used (Lemke and Axel, 1985; Griffiths et al., 1989). Digoxigenin-labelled probes were transcribed using the Boehringer SP6/T7 transcription kit and manufacturer's instructions. Transcripts were hydrolysed to give an average probe length of 150 bases and used at a concentration of 2.5 ng/μl. Schwann cell precursors and neurons on coverslips were fixed in 4% paraformaldehyde for 20 minutes and then dehydrated from 30% ethanol to 70% ethanol. The cells were rehydrated, and followed by digesting in proteinase-K (1.25μg/ml) for 3-3.5 minutes at room temperature. After washing in PBS the cells were refixed in 4% paraformaldehyde and treated with 0.1 M triethanolamine acetate. After hybridisation with cDNA probe as mentioned above at 50^0 C for at least 18 hr, cells were washed in 4XSSC for 50 minutes at room temperature, followed by 5 minutes in RNase buffer then digested in RNase-A (20μg/ml) for 30 minutes at 37^0 C, and followed by washing in RNase buffer at 37^0 C for 30 minutes, 2XSSC at 45^0 C for 30 minutes, 0.1XSSC 30 minutes at 55^0 C and buffer 1 (Tris-HCl 100mM, NaCl 150mM, pH 7.5) for 5 minutes. Cells were then blocked in 1% milk in buffer 1 for 1 hr at room temperature, and incubated in polyclonal sheep anti-digoxigenin conjugated to alkaline phosphatase in 1% milk in buffer 1 for 1 hr. After washing in buffer-1 30 minutes and buffer-3 (Tris-HCl 100mM, NaCl 100mM, $MgCl_2$ 50mM, pH 9.5) 10 minutes, the hybridised cells were visualised by enzyme catalysed colour reaction using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt.

Immunoblotting

Neurons from newborn rat DRG were cultured in a 35 mm petri dish with defined medium containing 50 ng/ml NGF plus IGF-1 (13nM) for 20 hr. The neurons were seeded at 3-4X10^5 per dish. Schwann cells from newborn rat sicatic nerves were cultured in a 35 mm^2 petri dish at a density of 5X10^5 cells per dish in defined medium containing FGF-2 (180pM) plus IGF-1 (13nM) for 20 hr. 2X10^5 Schwann cell precursors from E14 rat were cultured in defined medium containing FGF-2 (180pM) plus IGF-1 (13nM) for 20 hr. Proteins were extracted from the cells described above or from brain tissue by using 50-100 µl of 2% SDS in 5 mM Tris-Cl, pH 6.8, containing 2 mM EGTA, 2 mM EDTA, and 2 mM PMSF per dish. 20µg of each sample was applied to a 8% polyacrylamide gel. The separated proteins were then transferred to nitrocellulose, a molecular weight markers, SDS 6H, were used to calibrate the gel. The nitrocellulose sheet was blocked with 5% milk in PBS and then exposed to NDF antibody 5D6A (5 µg/ml) in 1% milk in PBS overnight at 4^{0}C. Rabbit anti-mouse Ig conjugated to biotin was applied for 1 hr followed by Vectastain Elite ABC kit for 30 minutes, made up according to manufacturers instructions. Bound antibody was detected using enhanced chemiluminescence (ECL) and X-ray film (X-Omat).

Culture of hybridoma cell lines

In order to obtain the anti L1, Thy 1.1 and 04 antibodies, the ASCS4 cell lines, OX-7 cell lines and 04 cell lines were cultured in 75 cm^2 flasks with 20-25 ml of hybridoma medium containing 10% fetal calf serum, 100mM glutamine, 100mM pyruvate, 10,000 IU/ml penicillin and streptomycin and 0.1 M 2-mercaptoethanol in RPMI. The supernatant was collected every other day, and stored at -20^{0}C until required.

Quantification

All quantitative results are based on a minimum of three separate experiments. Each determination within an experiment is based on counts from three coverslips in the majority of cases, but occasionally on counts from two coverslips. The error bar on graphs indicates SEM.

CHAPTER 3

THE SCHWANN CELL PRECURSOR: SURVIVAL AND PROLIFERATION

Introduction

The Schwann cell precursor, a distinct intermediate cell between neural crest cells and Schwann cells defined by previous work in our group, faces a life or death fate during development. In rat, Schwann cell precursors have been isolated from E14-E15 peripheral nerve (Jessen and Mirsky, 1992; Jessen et al., 1994). In vitro almost all Schwann cell precursors undergo apoptosis in 20 h in routine culture medium. The survival rate of these cells can be dramatically increased by the use of neuronal conditioned medium (Jessen et al., 1994), suggesting that neuronally derived molecules are crucial for the survival of these cells. In order to identify the molecules necessary for Schwann cell precursor survival many growth factors were screened in the short-term survival assay. It was found that FGFs combined with IGF could rescue 100% of the Schwann cell precursors from apoptosis in 20 h cultures (Jessen et al., 1994; Gavrilovic et al., 1995). However, for long-term survival, it was necessary to add serum in addition to FGF and IGF, suggesting that some unidentified molecules may act as a long-term survival factors for Schwann cell precursors.

Schwann cell precursors in vivo normally undergo vigorous proliferation (Stewart et al., 1993). But so far no molecules have been identified as mitogens for Schwann cell precursors in vitro. FGF-2 plus forskolin, a typical mitogen for Schwann cells, fails to induce DNA synthesis in Schwann cell precursors (Garvrilovic et al., 1995). But in birds and rats, FGFs in the presence of serum and embryo extract stimulates neural crest cell proliferation (Bannerman and Pleasure, 1993; Murphy et al., 1994). Therefore, some unidentified molecules may exist in serum or embryo extract which synergize with FGF or act by themselves alone to trigger Schwann cell precursor proliferation. As mentioned previously, members of the novel growth factor family, NDF\ GGF\ HRG, have recently been found to be expressed at high levels in embryonic spinal cord and dorsal root ganglia (Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994). These molecules have also been shown to have a potent mitogenic effect on Schwann cells (Marchionni et al., 1993; Goodearl et al., 1993; Stewart et al., 1995). Therefore it is of interest to know the role of these molecules in Schwann cell precursor development.

In early studies of Schwann cell proliferation, it was found that bovine pituitary extracts had ability to stimulate Schwann cell proliferation (Raff et al., 1978; Brockes et al., 1979), which led to the partial purification of a new growth factor named glial growth factor (GGF) (Lemke and Brockes, 1984). Recent purification and cDNA cloning of this molecule led to the discovery that GGF belongs to a novel growth factor family including NDF, HRG, GGF and ARIA (Ben-Baruch and Yarden, 1994). The biological activity of NDFs has been shown to promote both proliferation and differentiation depending upon the cell type (Peles et al., 1992; Falls et al., 1993; Goodearl et al., 1993). In the Schwann cell lineage, NDF has been shown to stimulate Schwann cell DNA synthesis both with and without cAMP elevation (Goodearl et al., 1993; Marchionni et al., 1993; Stewart et al., 1995). In the early stages of development, NDF is able to influence the lineage choice of neural crest cells in vitro. (Shah et al., 1994). This implies that NDF may act as a key molecule in the development of the Schwann cell lineage.

NDF is known to activate its biological events by binding to specific receptors. The identified receptors for NDF are ErbB2, ErbB3 and ErbB4. It is clear that NDF can phosphorylate all three receptors when these receptors are co-expressed in cells, although the phosphorylation of the receptor is quite complicated when the receptor is expressed on its own (Tzahar et al., 1994 Sliwkowski et al., 1994; Carraway et al., 1994; Carraway and Cantley, 1994). Both ErbB2 mRNA and protein have been shown to be highly expressed on Schwann cells at the early postnatal stage and their expression decreases significantly up to adulthood (Cohen et al., 1992; Jin et al., 1993). More recently, ErbB2 and ErbB3 have also been detected in neural crest cells (Shah et al., 1994; Meyer and Birchmeier, 1995). But so far there is have been no reports showing the expression of ErbB4, the more recently discovered NDF ligand binding receptor, in cells of the Schwann cell lineage.

The results in this chapter show that NDFβ isoforms but not NDFα isoforms act as survival factors for Schwann cell precursors in defined medium at the pM level. The survival activity of NDFβ depends on its EGF-like domain. This survival activity of

NDFβ can also be blocked by polyclonal anti-NDF antibody. In a long term survival assay, NDFβ not only rescues Schwann cell precursors from programmed cell death for several days but also maintains the response of these cells to FGF. Furthermore, IGF is shown to significantly increase the survival activity of NDFα. In contrast, neither IGF nor insulin is required for the effect of NDFβ in promoting Schwann cell precursor survival. NDFβ is so far the only known growth factor that acts as a mitogen for Schwann cell precursors when applied on its own. TGFβ in combination with FGF and forskolin and IGF-1 can also stimulate Schwann cell precursor DNA synthesis. In addition the present study also shows that Schwann cell precursors express the NDF receptors ErbB2 and ErbB4. Together, these observations indicate that NDF may play a important role in Schwann cell precursor development.

Results

NDFβ acts as a survival factor for Schwann cell precursors in short term cultures

In order to understand the effects of NDF in Schwann cell precursor survival assays, various doses of NDFβ–2 and NDFα–2 were used in defined serum free medium containing a low concentration of insulin (1nM) for 20 h. In this entirely defined medium only insulin receptors but not IGF type I receptors will be activated due to very low insulin levels (Sara and Hall, 1990; Neely, 1991). The results showed that even at the pM level NDFβ–2 markedly increased the survival rate of Schwann cell precursors (Figure 3.1). The dose-dependent survival curve indicated that maximal survival (100%) occured between 40-120pM. Without NDFβ essentially all precursors were dead by 20 hr (Figure 3.2). In contrast to NDFβ–2, NDFα–2 had little survival activity for precursors even at the nM level in this low insulin medium.

To further test whether different domains of NDFs might affect survival, the NDFβ-1 or NDFβ-3 which differ from NDFβ-2 in the amino acid sequence in the juxtamembrane region (Wen et al., 1994), were tested in the same conditions as described above. The results indicated that both NDFβ-1 and NDFβ-3 had a similar effect on survival to NDFβ-2 (Figure 3.3A), while the corresponding α isoforms were ineffective. Furthermore, the Schwann cell precursors were also challenged with NDF-EGFβ-1 which lacks immunoglobulin domain and the spacer domain, and NDF-EGFβ which only has the EGF-like domain. It was found that the polypeptide containing only the EGFβ domain of 40 amino acids was sufficient to rescue all precursors from death (Figure 3.3B).

IGF-1 promotes the survival activity of NDF

In previous studies from our group, IGF was thought to be an essential factor in mediating the FGF rescue of Schwann cell precursors. The survival activity of FGF appeared to depend on activation of IGF type I receptors (Gavrilovic et al., 1995). NDFβ as shown above, was able to promote precursor survival in the low insulin and no IGF, indicating that IGF type I receptor activation is not necessary for NDFβ mediated precursor survival. However, NDFα in the same medium had very little survival promoting activity. Therefore, experiments were performed to test whether IGF was necessary for NDFα to mediate survival and whether it would enhance NDFβ survival activity in this assay. It was found that IGF-1 (13nM) did not significantly shift the NDFβ–2 survival curve. Surprisingly, when NDFβ–2 was used in the absence of both insulin and IGF medium the survival of Schwann cell precursors could still reach 100% at pM concentrations of NDFβ–2, but the ED_{50} (the concentration giving half maximal survival) was 5-6 fold higher than that seen in low insulin medium (Figure 3.4A). In contrast to NDFβ-2, IGF-1 markedly enhanced NDFα-2 mediated survival. The maximal survival rate of precursors in the presence of both IGF-1 (13nM) and NDFα-2 (4 nM) reached 70% (Figure 3.4B).

Since IGF-1 has been shown to greatly enhance the survival activity of NDFα-2, the same experiment was also done using NDFα-1 and NDFα-3. It was found that both NDFα-1 and NDFα-3 in the presence of IGF-1 (13nM) had a similar survival activity as NDFα-2 (Figure 3.5A). Furthermore, NDF-EGFα-2 which has an α EGF-like domain and juxtamembrane region also showed some survival potential but less than that of the whole α isoform in the presence of IGF-1 (13nM). Thus the limited survival potential of NDFα may also partially depend on its α–EGF-like domain (Figure 3.5B).

NDF induces DNA synthesis in Schwann cell precursors

Before testing the mitogenic activity of NDFβ on Schwann cell precursors it was noticed that the survival rate always overshot 100% when high concentrations of

NDFβ had been applied in the medium (Figure 3.1). This suggests that NDFβ may act as a mitogen for Schwann cell precursors as well as for Schwann cells.

Two separate experiments performed in the presence of IGF-1 (13nM) were devised to test this. First, in a 20 hr survival assay, BrdU was introduced into the medium three times, with 1.5 hr long pulses at each time point. The BrdU reaction was ended at 5 hr, 15 hr and 20 hr after plating. DNA synthesis was monitored in L1 positive cells with BrdU nuclear labelling. It was found that in the presence of FGF-2 (180pM) plus forskolin the proliferation of Schwann cell precursors fell rapidly from 5 h (13.67%) to 20 h (0.97%). In contrast, in the presence of NDFβ-2 the division of precursors varied with different concentrations of NDFβ-2. In high concentrations of NDFβ-2 (400pM) DNA synthesis increased from 5 h (13.88%) to 20 h (22.54%). But in low concentrations the division pattern is similar to that of FGF-2 plus forskolin (Figure 3.6A). Interestingly it was found that in the 5 h assay DNA synthesis of precursors in all experimental groups was about 13% which excellently matched previous experiments in vivo (Stewart et al., 1993).

In additional experiments, NDFβ-2 was used in conditions in which Schwann cell precursors had fallen out of cell division. Schwann cell precursors were first cultured in FGF-2 medium for 20h, then changed to NDFβ-2 medium for another 20 h. BrdU was introduced into the medium for only 1.5 hr at each time point and the incubation were terminated at 5 hr, 15 hr, 20 hr, 25 hr and 40 hr respectively. Under these conditions NDFβ-2 still stimulated DNA synthesis of Schwann cell precursors to 24.16% at the 40 hr point although at the 20 hr point the division rate of precursors was only 1% in FGF-2 medium (Figure 3.6B). The DNA synthesis rate at the 40 hr (24.16%) in this experiment is similar to that in the 20 hr experiment (22. 54%) when NDFβ-2 was added directly after plating (Figure 3.7).

In the experiments described above, the different NDFβ isoforms were shown to have similar effects on the survival of Schwann cell precursors. The different isoforms were also used for mitogenic assays. Only the 20 hr assay was carried out in this

experiment and BrdU was added for the last 1.5 hr of a total 20 hr period. It was found that all NDFβ isoforms have the same effect on DNA synthesis of precursors (Figure 3.8A). Moreover, NDF-EGFβ-1 and NDF-EGFβ, as described before for survival, also stimulated DNA synthesis of these cells and were as potent as NDFβ–2 (Figure 3.8B). Since high concentrations (4nM) of NDFα–2 could still support 70% of Schwann cell precursor survival. DNA synthesis of precursors at this concentration was also tested, but only 0.88% of cells were BrdU positive. All experiments described above were carried out in the presence of IGF-1 and it was shown that all NDFβ isoforms are dose-dependent mitogens for Schwann cell precursors, while NDFα is not. Since NDFβ is largely independent of IGF for support of precursor survival NDFβ was also used in only low insulin defined medium for precursor division assays. It was found that the mitogenic dose-response curve in the absence of IGF was quite similar to that in the presence of IGF (Figure 3.9) although IGF seemed to slightly enhance the mitogenic activity of NDFβ.

TGFβ stimulates Schwann cell precursor DNA synthesis in the presence of both FGF and IGF

In the experiments above, NDFβ acts as a dose-dependent mitogen for Schwann cell precursors. In order to identify other possible mitogenic molecules for Schwann cell precursors many growth factors were screened in the presence of FGF-2, since all growth factors applied in these experiments were previously found to be unable to support Schwann cell precursor survival (Jessen et al., 1994). FGF is the only molecule which is known to support survival without induction of DNA synthesis in Schwann cell precursors. Eight different growth factors including TGFβ–1, TGFβ–2, PDGF-BB, PDGF-AA, EGF, NGF, BDNF and NT-3, were added on top of medium containing FGF-2 (180pM), IGF-1 (6.5nM) and forskolin (5μM) (referred to henceforth as FIF medium). BrdU was applied during the last 2-4 h of a total 20 h assay to measure DNA synthesis. The results showed that only TGFβ stimulated Schwann cell precursor DNA synthesis under these culture conditions (Table 3.1).

Addition of TGFβ (1ng/ml) to the FIF medium gave rise to 20% DNA synthesis of Schwann cell precursors in comparison to 1.8% in FIF medium alone (Figure 3.10A). Dose-response curves for the mitogenic activity of TGFβ were generated in a 20 h BrdU assay on both E14 and E13 Schwann cell precursors. It was found that addition of 0.1 ng/ml of TGFβ on top of the FIF medium caused DNA synthesis in more than 90% of precursors (Figure 3.10B). Meanwhile, control experiments showed that without FGF in this growth factor cocktail all precursors underwent programmed cell death. This suggests that TGFβ acts only as a mitogen in the presence of other factors and has no survival effect on precursors.

Identification of L1 as a specific marker of Schwann cell precursors

In previous studies p75NGF-R was used as specific marker for Schwann cell precursors (Jessen et al., 1994; Gavrilovic et al., 1995). At the beginning of the survival experiments described above, p75NGF-R staining was also used. It was found that a small population of the p75NGF-R positive cells showed distinctive characteristics which included a more bipolar morphology in comparison to the flattened morphology of Schwann cell precursors, and isolation as single cells in comparison to the group-forming tendency of p75NGF-R positive precursors. In FGF-2 medium a lot of these isolated bipolar cells were BrdU positive, whereas cells in the flattened grouped cells were not. After screening several antibodies, L1 was shown not to label these cells although it bound to cells of the other p75NGF-R positive cells in double label experiments (see below). Together, the evidence indicates that this small population of cells tend to lie isolated, have a bipolar phenotype, divide in FGF-2 medium and are L1 negative (Figure 3.11). It is possible that these p75NGF-R^{+}, $L1^{-}$ cells do not belong to the Schwann cell lineage. In order to monitor the effect of this small population of cells in the survival and proliferation assays, p75NGF-R, L1 and BrdU triple labelling was used in the medium containing either FGF-2 or NDFβ-2. Three separate BrdU doses were applied in the 20 h assay (as above). It was found that all L1 positive cells were p75NGF-R positive, whereas a few p75NGF-R positive cells were L1 negative. In FGF-2 medium BrdU positive, L1

positive cells decreased rapidly, whereas BrdU positive, p75NGF-R positive cells still remained at 10% at 20 hr (Figure 3.12A). These data implied that FGF-2 might only stimulate DNA synthesis in $L1^-$, $p75NGF\text{-}R^+$ cells. In NDFβ-2 medium, only 3% of p75NGF-R positive cells were found to be L1 negative, and the outcome of this division assay did not significantly depend on whether L1 or p75NGF-R were used (Figure 3.12B). Thus, the use of either L1 marker or p75NGF-R labelling would lead to similar results in the precursor survival assay. Nevertheless, all the proliferation assays described above and the long-term survival assay described later in this chapter were carried out using L1 labelling, and in the short-term survival assay most experiments were confirmed by L1 labelling although some of the early experiments began used the p75NGF-R marker. The evidence suggests that L1 is a more specific marker for Schwann cell precursors than p75NGF-R.

NDF supports long term survival of Schwann cell precursors

NDFβ has been shown to support Schwann cell precursor survival in a 20 h assay (shown above), and FGF-2 was also reported as a survival factor in short term cultures in previous studies (Jessen et al., 1994; Gavrilovic et al., 1995). To further test whether these molecules can also support the long term survival of precursors, NDFβ-2 and FGF-2 were applied in low insulin defined medium with IGF-1 (13nM). Meanwhile, in order to limit the effect of proliferation of precursors in this assay, a low concentration of NDFβ-2 (32pM) only was used in all these experiments. The survival assay was carried out at the 20 hr, 44 hr and 68 hr, and total surviving cells were counted using L1 labelling. It was found that in NDFβ medium the total number of cells at the 68 hr point was higher than that at the 20 hr point (Figure 3.13B). Using BrdU to label precursors at the 20 hr and 44 hr it was found that about 3% of cells were synthesizing DNA in the 1.5 h BrdU assay. It implies that this small division may generate a small increase in the total number cells at 44 hr and 68 hr. In contrast to NDFβ, in FGF-2 there was a dramatic decrease of precursor survival after 20 hr, and at 68 hr less than 10% of cells survived (Figure 3.13A). This suggested that NDFβ is a long-term survival factor, whereas FGF-2 is not.

To further understand why Schwann cell precursors died after 20 h in FGF-2 medium, Schwann cell precursors were cultured in FGF-2 medium for 20 h, then cells were washed several times and changed to NDFβ-2, NDFα-2 or fresh FGF-2 medium respectively. The results showed that all precursors survived after being changed from FGF-2 to NDFβ-2 (Figure 3.14A), whereas, massive cell death was found in the remaining FGF-2 medium after another 20 h as shown above (Figure 3.13A). The survival rate in NDFα-2 medium was similar to that previously show in the 20 h assay using NDFα-2 (Figure 3.14B). These results indicated that NDFβ could still rescue precursors even after they had been exposed to FGF-2 for 20 h. It seems that precursors gradually lose responsiveness to FGF-2 in FGF-2 medium. However, FGF-2 maintains the response of precursors to both NDFβ and NDFα.

Since FGF-2 maintains the response of precursors to NDFβ and NDFα, but not FGF itself, it was of interest to know whether NDFβ can also maintain responsiveness of precursors to FGF. The experiment designed for this purpose was carried out under the same conditions as above. The Schwann cell precursors were cultured in NDFβ-2 (32pM) for the first 20 hr or 44 hr respectively, then changed to FGF-2 (180pM) medium. It was found that after 20 hr in NDFβ-2 the survival rate of precursors in response to FGF-2 over the next 24 hr was 83.61% (Figure 3.15A), which is similar to the precursors in FGF-2 in the 20 h assay (Figure 3.13A). Furthermore, after being in NDFβ-2 for 44 h Schwann cell precursors can totally survive in FGF-2 medium (Figure 3.15B). Therefore, these experiment indicate that NDFβ is able to maintain the survival response of precursors to FGF.

The experiments above showed that NDFβ-2 supports Schwann cell precursor survival for more than three days. It is known that E14 Schwann cell precursors develop into Schwann cells during three day period (E17 Schwann cells) in vivo (Jessen et al., 1994). Therefore it was of interest to know whether Schwann cell precursors under these culture conditions would also develop into Schwann cells.

Previous studies showed that Schwann cells differ from Schwann cell precursors by showing surviving well in routine medium and by expressing S100 protein (Jessen and Mirsky, 1991; 1992). To test whether Schwann cell precursors undergo maturation in NDF containing medium, precursors were cultured in NDFβ-2 for 20 h or 44 h respectively, then changed to low insulin defined medium without any specific survival factors, since only Schwann cells but not precursors can survive in this medium. The results showed that 52% of cells survived in this medium after the cells were exposed to NDFβ-2 for 2 days although most precursors died in this medium after cells had been exposed to NDFβ-2 for only 20 hr (Figure 3.16). Interestingly, the survival rate of 52% in this defined medium after 2 days in NDFβ-2 (which is equivalent to E 14 + 2 d = E16 in vivo) is similar to the survival rate of cells from E16 nerves in defined medium (Jessen et al., 1994). Meanwhile, a pilot study showed that Schwann cell precursors cultured in NDFβ-2 medium for more than 3 days expressed S-100. Parallel studies by A. Brennan give more detail and precise evidence to show that NDFβ drives Schwann cell precursor maturation (Dong et al., 1995).

The survival activity of NDF can be blocked by polyclonal anti NDF antibody.

To further determine the specificity of NDFβ survival activity in Schwann cell precursors, two antibodies were used. 1915#, a polyclonal antibody raised in rabbit, was originally thought to recognize NDFα specifically. It is now known to recognize sequences present in NDFβ as well (Personal communication from Dr. D. Wen Amgen Inc.). 114A, a monoclonal antibody, was designed to specifically bind to the c-loop of β EGF-like domain and therefore recognizes only NDFβ isoforms (Personal communication from Mr. D. Chang Amgen Inc.). The experiments were carried out in medium containing low insulin plus IGF-1 (13nM). Different concentrations of antibodies were mixed with NDFβ-2 (40pM) in the above medium and were kept for at least 2 h before the mixture was applied to the precursors. It was shown that 1915# antibody at 10μg/ml concentration significantly diminished the survival activity of

NDFβ-2 (Figure 3.17A). On the other hand, 114A antibody even at high concentration (50μg/ml) only reduced NDFβ-2 survival activity by 48% (Figure 3.17B). Both antibodies were also tested in FGF-2 medium, where no block of survival was detected, indicating that the antibodies do not cause non-specific toxic effects. These experiments indicated that the survival activity of the molecules which have been used the above experiments were due to NDF molecules, since their activity could be blocked by its corresponding antibody. Furthermore, the observation that blocking of the c-loop of β EGF-like domain did not totally demolish survival activity of NDFβ indicates that the biological function of NDFβ may depend on the whole EGF-like domain rather than just on the c-loop which contains the key sequence difference between α isoforms and β isoforms (Wen D et al., 1994).

Schwann cell precursors express NDF binding receptors ErbB2 and ErbB4

The results so far indicated that NDF not only supported Schwann cell precursor survival but also stimulated precursor DNA synthesis and probably promoted maturation of these cells. It is known that the ligand binding receptors for NDF are ErbB2, ErbB3 and ErbB4 (Carraway and Cantley, 1994). Therefore, it was important to know whether precursors also express these receptors to which NDF can bind to trigger survival and proliferation events. The experiment was carried out by applying anti ErbB2 and ErbB4 antibodies. Schwann cell precursors were cultured in both FGF-2 plus IGF and forskolin medium and NDFβ–2 plus IGF-1 medium for 20 h. After fixation in 4% paraformaldehyde for 20 minutes Schwann cell precursors were stained with either anti ErbB2 (1μg/ml) or anti ErbB4 (1μg/ml) antibodies. It was found that almost all precursors showed membrane-associated immunolabelling in both antibodies, although the intensity of immunolabelling of ErbB2 was more variable (Figure 3.18). The corresponding control experiment showed that ErbB4 control peptide abolished ErbB4 immunolabelling but not ErbB2 labelling, whereas ErbB2 control peptide diminished ErB2 immunolabelling but not ErbB4 labelling. This indicated that the staining applied here is a specific labelling for both ErbB2 and ErbB4, and therefore that Schwann cell precursors expressed both of these receptors.

Discussion

The experiments presented here showed that proteins of the novel growth factor family, NDF\GGF\HRG|ARIA, were able to support either short-term or long-term survival of Schwann cell precursors at pM concentrations without any other growth factors or serum. Neither IGF nor insulin is necessary for NDF to promote the survival of these cells. NDF not only rescues precursors from apoptosis but also stimulates the DNA synthesis of these cells at higher concentrations. In contrast, FGF acts as a short-term survival factor in combination with IGF, but it can neither support precursor long-term survival nor stimulate DNA synthesis in these cells. Whereas, TGFβ has been found to promote Schwann cell precursor DNA synthesis in the presence of FGF-2, forskolin and IGF-1, it has no survival activity at all for precursors on its own. The ability of NDF to promote survival, proliferation and maturation of Schwann cell precursors suggests that NDF may activate multiple signalling pathways in these cells.

NDF supports Schwann cell precursor survival

For Schwann cell precursors NDF on its own seems sufficient for both short-term and long-term survival. This survival effect was in completely defined media and the cells used in these assays had never been exposed to serum or any other unidentified factors during either dissociating procedure or in culture. Meanwhile the rescue required neither IGF-1 nor insulin, indicating that NDF is distinctly independent of any other factors to support Schwann cell precursor survival.

It is known that a low concentration of insulin (10nM) only activates the insulin receptor, while 13nM IGF-1 would activate both the insulin and IGF type-1 receptors; high concentrations of insulin can mimic the effect of IGF-1 and activate both IGF type-1 receptors and insulin receptors (Sara and Hall, 1990; Neely et al., 1991). FGF, unlike NDFβ, depends on either IGF or high concentrations of insulin to

promote Schwann cell precursor survival, indicating that both IGF type-1 and insulin receptor activation is necessary for FGF mediated precursor survival (Gavrilovic et al., 1995). Higher concentrations of NDFβ-2 (400pM) support total precursor survival in the absence of both IGF and insulin. This implies that neither insulin receptor nor IGF type-1 receptor activation is necessary for NDF mediated Schwann cell precursor survival.

In addition to being independent of IGF, NDF promotes the long-term survival of these cells, while FGF only supports precursor survival for 20 hr. The rapid loss of survival of precursors during the 2nd and 3rd days in FGF-2 medium does not reflect a normal developmental change of these cells in vivo, since FGF-2 supports survival of cells directly dissociated from E15 or E16 nerves (Jessen et al., 1994). Meanwhile, it is also clear that Schwann cell precursors in FGF-2 medium for 20 hr are not irreversibly set on a death course, since NDFβ was shown to rescue all precursors which have been exposed to FGF-2 medium for 20 hr. Moreover, previous experiments show that FGF can support the long-term survival of precursors only when FGF is combined with some unidentified factors in serum (Jessen et al., 1994). Therefore, it is clear that Schwann cell precursors in vivo and in serum containing medium in vitro maintain full responsiveness to FGF, whereas precursors gradually lose the ability to respond to FGF in serum free medium containing FGF-2. It is probable that some unidentified molecules in vivo or serum may preserve the responsiveness of precursors to FGF. We have then taken a further step in this direction of identifying such molecules by showing that NDF maintains full FGF-2 responsiveness in vitro. Although the molecular basis by which NDF regulates the response to FGF is not clear, one possibility is that NDF may regulate FGF receptor expression by precursors. The observation that responsiveness to NDF is maintained in vitro may reflect NDF regulation of its own receptors, since NDF\GGF has been shown to upregulate ErbB2 (neu) mRNA in Schwann cells (Cohen et al., 1992).

Proliferation of Schwann cell precursors

Although it is clear that Schwann cell precursors in E14 or E15 nerves in vivo undergo proliferation (Stewart et al., 1993) the molecular basis for this is unknown. It has been shown previously that FGF plus IGF and forskolin, a routine mitogen for Schwann cells of all ages in culture, failed to stimulate Schwann cell precursor proliferation (Jessen et al., 1994; Gavrilovic et al., 1995). These results indicated that Schwann cells and Schwann cell precursors might have different responses to mitogens. The experiments presented here showed that NDFβ and TGFβ are mitogens for precursors in vitro, but the two growth factors showed significant differences in the conditions they require if they are to stimulate DNA synthesis.

NDF, in the present study , showed a dose-dependent stimulation of Schwann cell precursor DNA synthesis in defined medium, and this effect is seen in the absence of forskolin or cAMP analogues. It is well known that most growth factors rely on cAMP elevation or cAMP analogues to trigger Schwann cell proliferation in serum free defined medium, suggesting that elevation of intracellular cAMP is necessary for Schwann cell division in most cases (Stewart et al., 1991). NDF, however, triggers DNA synthesis in Schwann cells also in the absence of cAMP elevation (Stewart et al., 1996). It is not clear how these findings relate to the observation that NDF/GGF has been reported not to elevate intracellular cAMP in 3T3 cells (Yoshimura et al., 1993). It is possible that NDF may activate the downstream components in cAMP pathway which could also be triggered by elevating cAMP. An alternative possibility is that NDF triggers a completely different pathway rather than the pathway triggered by cAMP.

In the present experiments, NDF also stimulated precursor proliferation in low insulin defined medium, implying that IGF type-1 receptor activation is not necessary for NDF mediation of Schwann cell precursor proliferation. This is somewhat surprising since activation of IGF type-1 receptor was thought to be essential for other growth factors to generate proliferation and differentiation events of Schwann cells (Stewart et al., 1996). In the CNS, in order to stimulate purified oligodendrocyte precursor proliferation it is essential to activate the IGF type-1 receptor, suggesting that the multiple signals are required for oligodendrocyte

proliferation (Barres et al., 1993; Barres and Raff, 1994). But NDFβ expresses a very strong mitogenic potential which promotes Schwann cell precursor DNA synthesis in the absence both of elevation of intracellular cAMP and activation of the IGF type-1 receptor, indicating that, at least in vitro, multiple factors are not always necessary for the proliferation of cells in Schwann cell lineage.

In contrast, TGFβ acts only as a mitogen for precursors in the presence of FGF-2, IGF and forskolin. Without either FGF or IGF, precursors will not survive in TGFβ alone. Therefore it is clear that TGFβ acts only as a mitogen and has no survival activity, whereas FGF functions only as a survival factor and has no mitogenic activity for precursors. In these cases the survival pathway and the mitogenic pathway in Schwann cell precursors appears to be triggered separately by two different growth factors. However, NDF can activate both pathways to promote precursor survival and proliferation, and this activation seems independent of both IGF and forskolin. The most likely explanation for this observation is that NDF may activate a broad spectrum of cytoplasmic signalling pathways in Schwann cell precursors, including those activated by cAMP, IGF, TGFβ and FGF.

The effect of different NDF isoforms

The two main isoforms of NDF, NDFα and NDFβ, have a significantly different biological activity in terms of survival and mitogenic activity for Schwann cell precursors. NDFβ gives rise to 100% precursor survival even in low or no insulin medium, whereas NDFα at the same concentration (40pM) only supports 12% precursor survival in the presence of both insulin and IGF. It is known that both NDFβ and NDFα can bind and phosphorylate ErbB3 and ErbB4, and when all three receptors, ErbB2, ErbB3 and ErbB4 are co-expressed in cells the phosphorylation of all three receptors has been observed (Tzahar et al., 1994; Sliwkowski et al., 1994; Scltoff et al., 1994). Meanwhile NDFβ isoforms have been shown to have an 8-10 fold higher binding affinity than that of NDFα isoform in cultured mammary cells

(Wen et al., 1994). Therefore, it is possible that increasing concentrations of NDFα may mimic the survival activity of NDFβ. However, when 10 fold or even 100 fold higher concentrations of NDFα (4nM) were tested for precursor survival in the presence of IGF, NDFα only give rise to 70% precursor survival while NDFβ at 40pM already promoted 100% precursor survival. Therefore, it seems likely that NDFβ may activate additional signalling pathways to those activated by NDFα which leads NDFβ to be a survival factor and also a mitogen for Schwann cell precursors.

In line with this, NDFβ-1 and β3-GGF-II have been shown to be the predominant isoforms in nervous tissue, whereas NDFα isoforms are thought of as the mesenchymal and nonneuronal isoforms (Meyer and Birchmeier, 1994; Wen et al., 1994). Thus, the expression of specific isoforms of NDF in a particular tissue may relate to its function in this tissue. As regards the question of which part of NDF domains acts as the functional domain in the survival and proliferation assays, the present study showed that differences in the juxtamembrane region which subdivides NDF into 1, 2, 3 and 4 sub-isoforms, do not generate differences in survival activity or mitogenic responses. It suggests that this region may not act as a functional domain. As suggested before, it may be involved in NDF secretion (Wen et al., 1994). The experiments here show that the β isoform EGF-like domain alone effectively triggered both survival and proliferation, in agreement with previous observations that the EGF-like domain is the functional domain of NDF(Wen et al., 1994). Interestingly, monoclonal antibody 114A which specifically recognizes the c-loop of the β-EGF-like domain fails to block NDFβ-2 activity completely, indicating that the whole EGF-like domain is necessary to trigger the biological activity of NDFβ although the c-loop of the EGF-like domain is the key sequence which distinguishes NDFβ from NDFα isoforms.

Co-expression of ErbB2 and ErbB4 in Schwann cell precursor

It is clear that NDF regulates cellular survival and proliferation by stimulating the intrinsic protein tyrosine kinase activities of their specific cell surface receptors, ErbB2, ErbB3 and ErbB4. When ErbB2 is co-expressed with either ErbB3 or ErbB4 NDF can phosphorylate both receptors. When ErbB2 is expressed in the cells on its own, no phosphorylation of ErbB2 occurs (Carraway et al., 1994; Sliwkowski et al., 1994; Tzahar et al., 1994). The present studies show that Schwann cell precursors express both ErbB2 and ErbB4 receptors. Since an anti rat ErbB3 antibody was not available when experiments were carrying out, this study was unable to determine whether ErbB3 is expressed by precursors, but one recent study showed that ErbB3 mRNA was also detected on neural crest cells and Schwann cell precursors of mouse suggesting that this receptor may also be involved in early development of cells in the Schwann cell lineage (Meyer and Birchmeier, 1995). Based on an analysis of amino acid sequences of ErbB2, ErbB3 and ErbB4 receptors it has been suggested that ErbB2, ErbB3 and ErbB4 might recruit many SH2 domain-containing proteins to activate a wide range of signalling pathways (Carraway and Cantley, 1994).

Table 3.1: List of mitogens for Schwann cell precursors

		In the presence of FGF-2 plus IGF and Forskolin	Growth factor alone
TGFβ-1	0.01-10ng/ml	+	no survival
TGFβ-2	0.01-10ng/ml	+	no survival
EGF	10ng/ml	-	no survival
PDGF-AA	4-10ng/ml	-	no survival
PDGF-BB	5-10ng/ml	-	no survival
NGF	50ng/ml	-	no survival
BDNF	10ng/ml	-	no survival
NT-3	10ng/ml	-	no survival
FGF-1	1-10ng/ml	n.d.	-
FGF-2	1-10ng/ml	-	-
FGF-4	20ng/ml	-	n.d.
NDFβ	1-30ng/ml	+	+
NDFα	1-100ng/ml	n.d.	-

n.d. Not determined.

Figure 3.1 NDFβ-2 prevents death of Schwann cell precursors

Schwann cell precursors obtained from E14 rat nerves were cultured in defined medium containing either NDFβ-2 or NDFα-2 for 20 hr. L1 antibody was used to label precursors after 20 hr. The number of precursors counted on coverslips at 20 hr is expressed as a percentage of the number of precursors present 3 hr after plating. This is referred to as the percent survival. The results show that NDFβ-2 acts as a dose-dependent survival factor for precursors, but not NDFα-2.

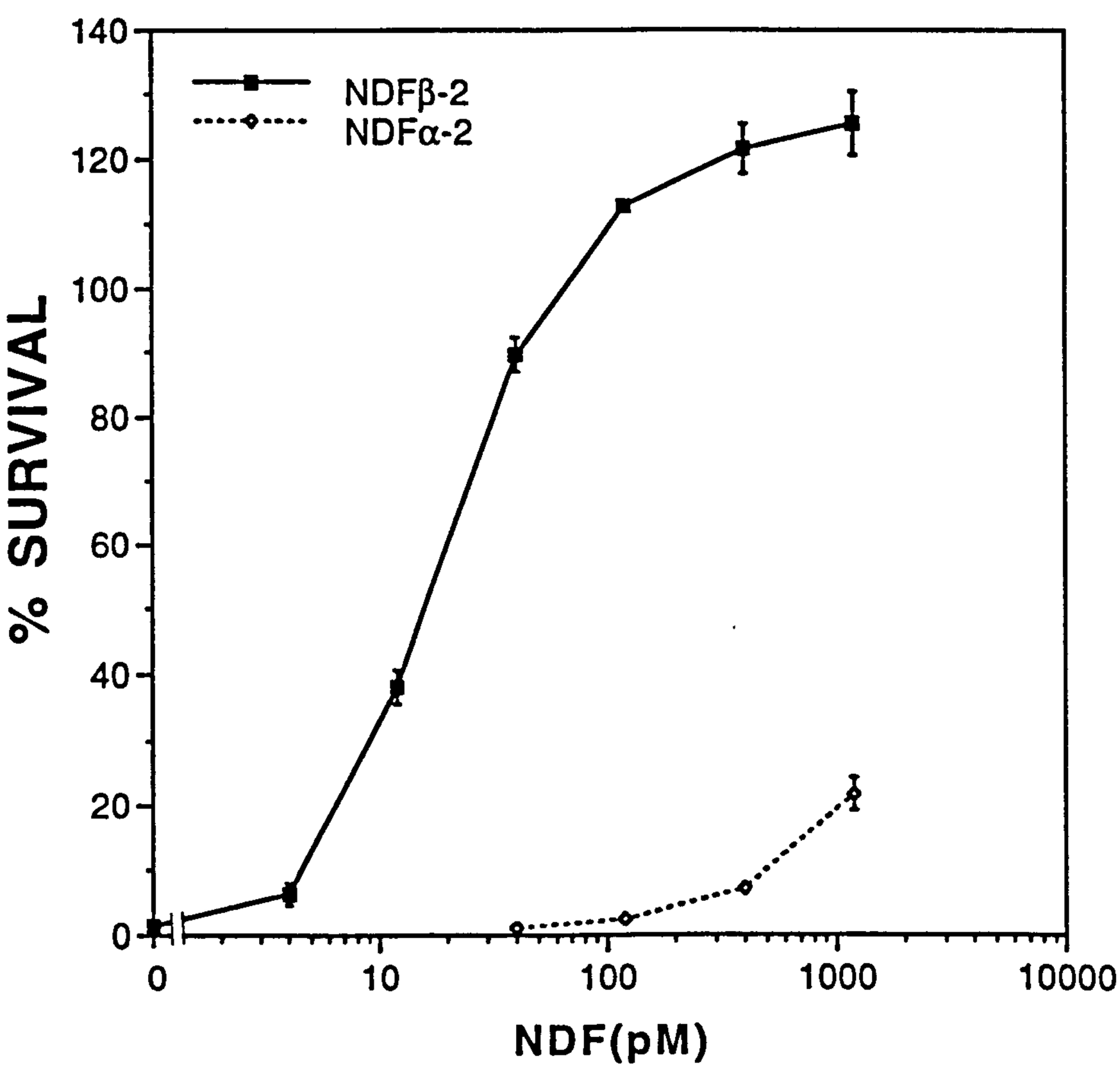

NDFβ-2
NDFα-2
% SURVIVAL
140
120
100
80
60
40
20
0
0
10
100
1000
10000
NDF(pM)

Figure 3.2 NDFβ supports precursor survival for 20 hr.

Schwann cell precursors dissociated from E14 sciatic nerves were cultured in defined medium or defined medium containing NDFβ-2 for 20 hr. At the 3 hr point, all precursors attached on coverslips (A). After 20 hr, precursors in defined medium only die (B), while precursors in NDFβ containing medium survive (C). The surviving precursors are shown by L1 labeling (D). Magnification 600X

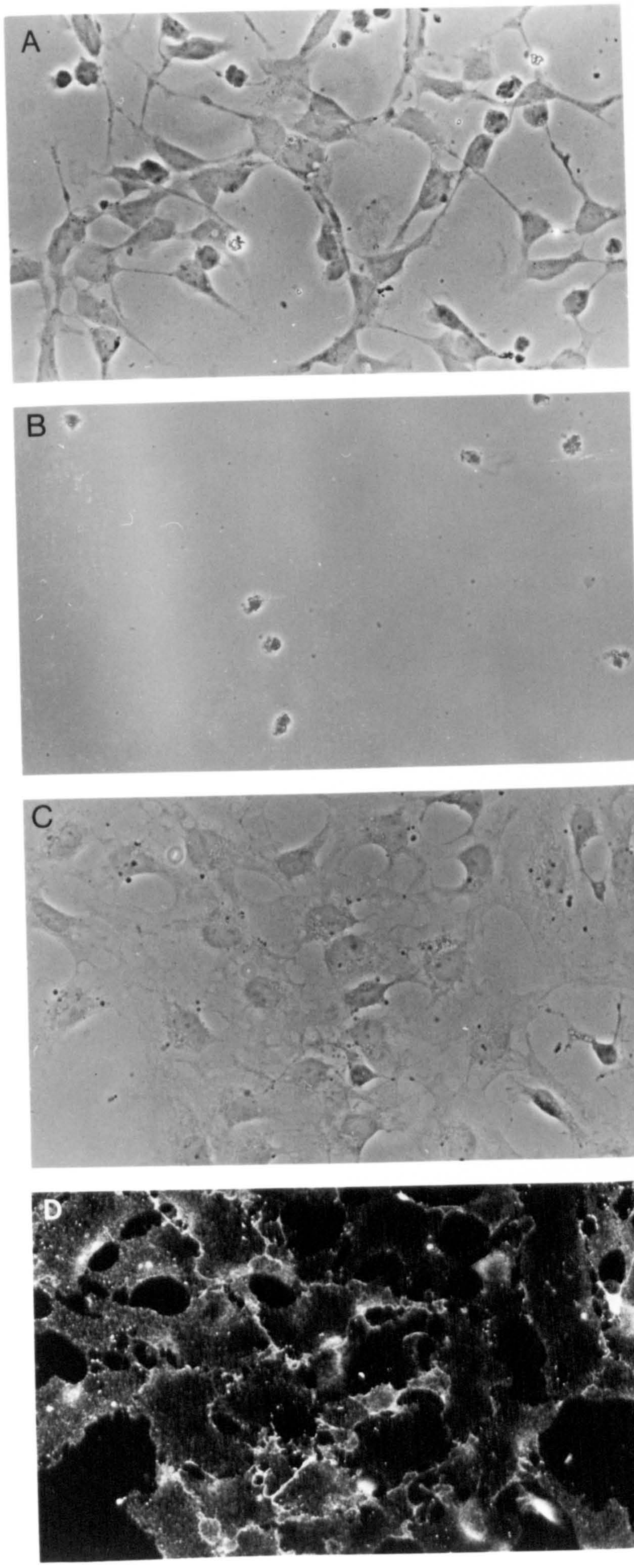
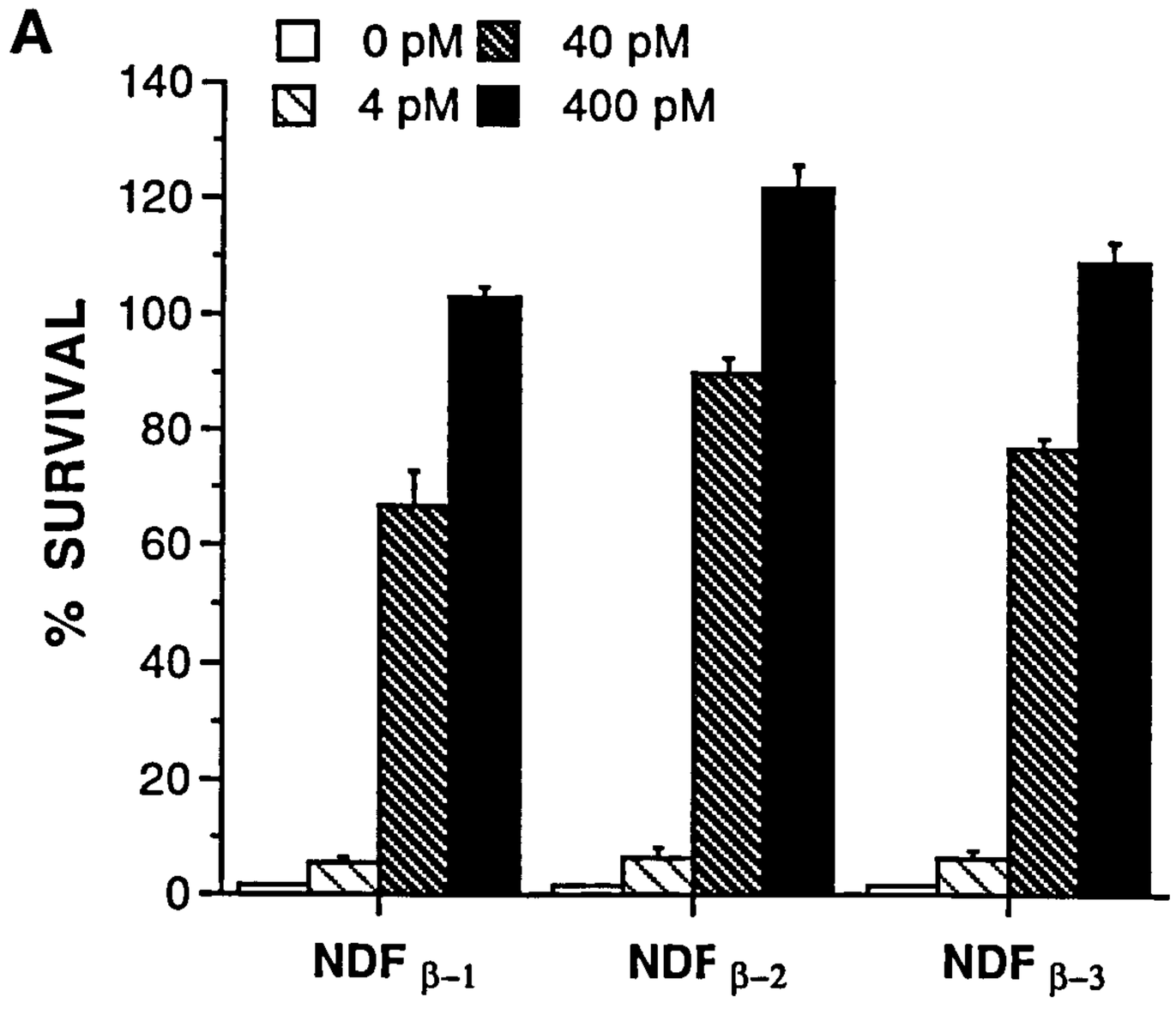

A
B
C
D

Figure 3.3 NDFβs support Schwann cell precursor survival

Schwann cell precursors were cultured in defined medium containing different isoforms of NDFβ (A), or NDF-EGFβ1 domain and NDF-EGFβ domain only (B) for 20 hr. The precursors were labeled with L1 antibodies. The results show that different NDFβ isoforms have similar activity in supporting precursor survival and that the EGFβ domain acts as the functional domain of NDFβ forms.


A
0 pM
40 pM
4 pM
400 pM
% SURVIVAL
140
120
100
80
60
40
20
0
NDF β–1
NDF β–2
NDF β–3

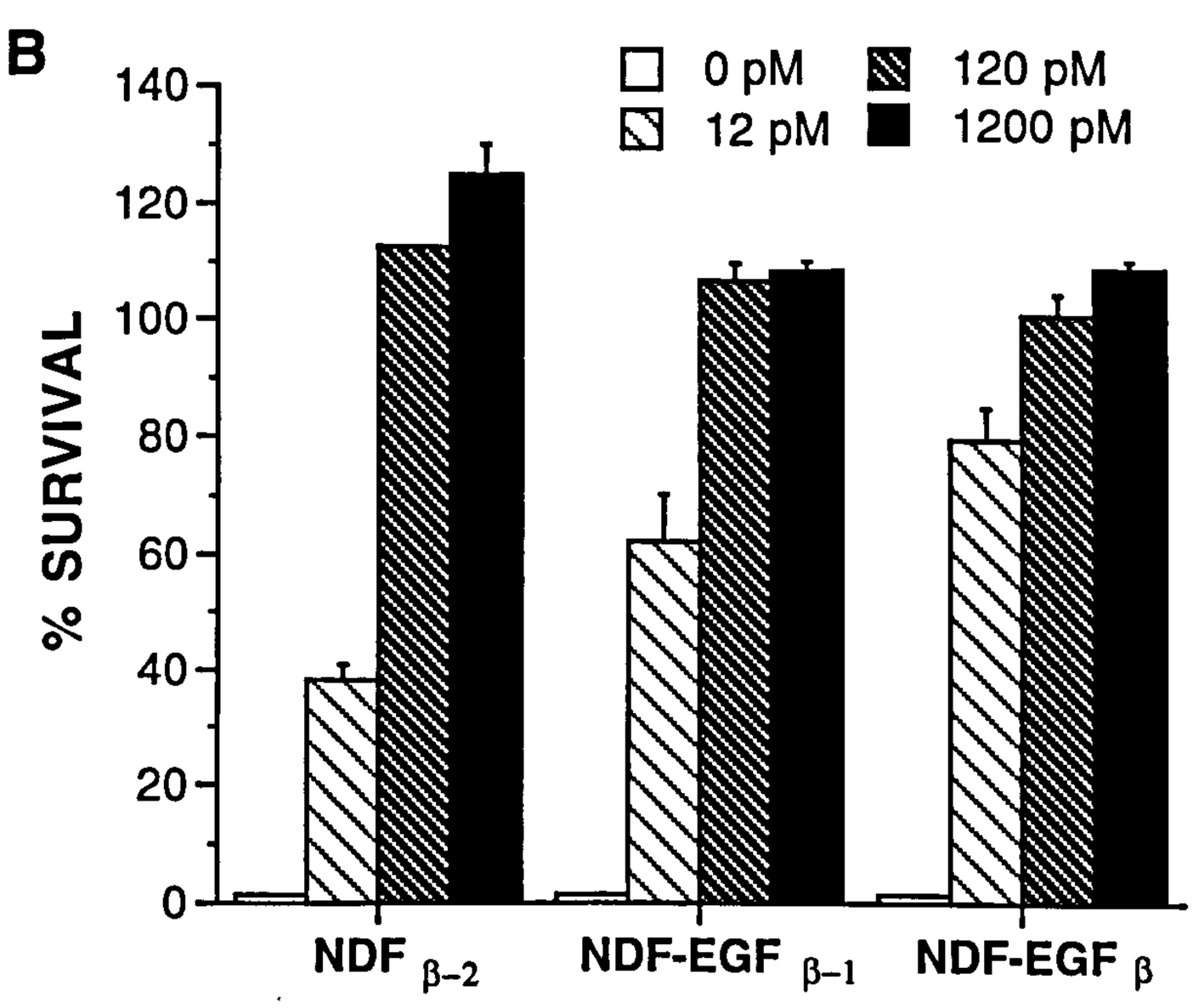
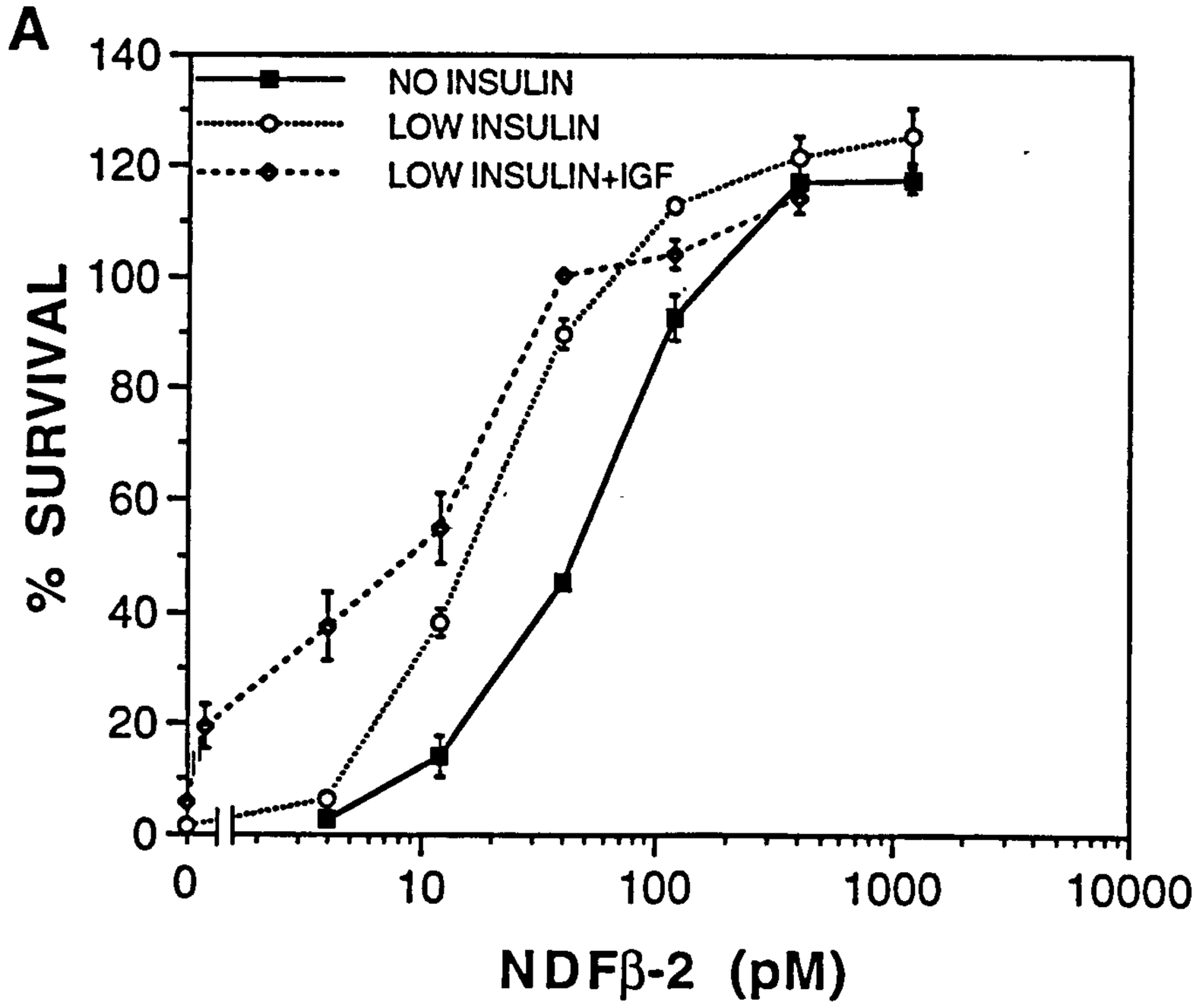

B
0 pM
120 pM
12 pM
1200 pM
% SURVIVAL
140
120
100
80
60
40
20
0
NDF β–2
NDF-EGF β–1
NDF-EGF β

Figure 3.4 Insulin-like growth factor promotes NDF mediated Schwann cell precursor survival.

Schwann cell precursors were cultured in defined medium containing no insulin, low insulin and low insulin plus IGF-1 (13nM), and the NDFβ-2 *dose-response curves were constructed on top of these media (A). NDFα-2 doses-response curves were based on defined medium containing low* insulin or low insulin plus IGF-1 (13nM) for 20 hr. L1 antibodies were used to label the surviving precursors. The results show that NDFβ *supports precursor survival in the absence of both insulin and IGF, and extra insulin and IGF shift the survival curve at low concentrations of* NDFβ. IGF significantly promotes NDFα mediated Schwann cell precursor survival.


A
140
120
100
80
60
40
20
0
% SURVIVAL
NO INSULIN
LOW INSULIN
LOW INSULIN+IGF
0
10
100
1000
10000
NDFβ-2 (pM)

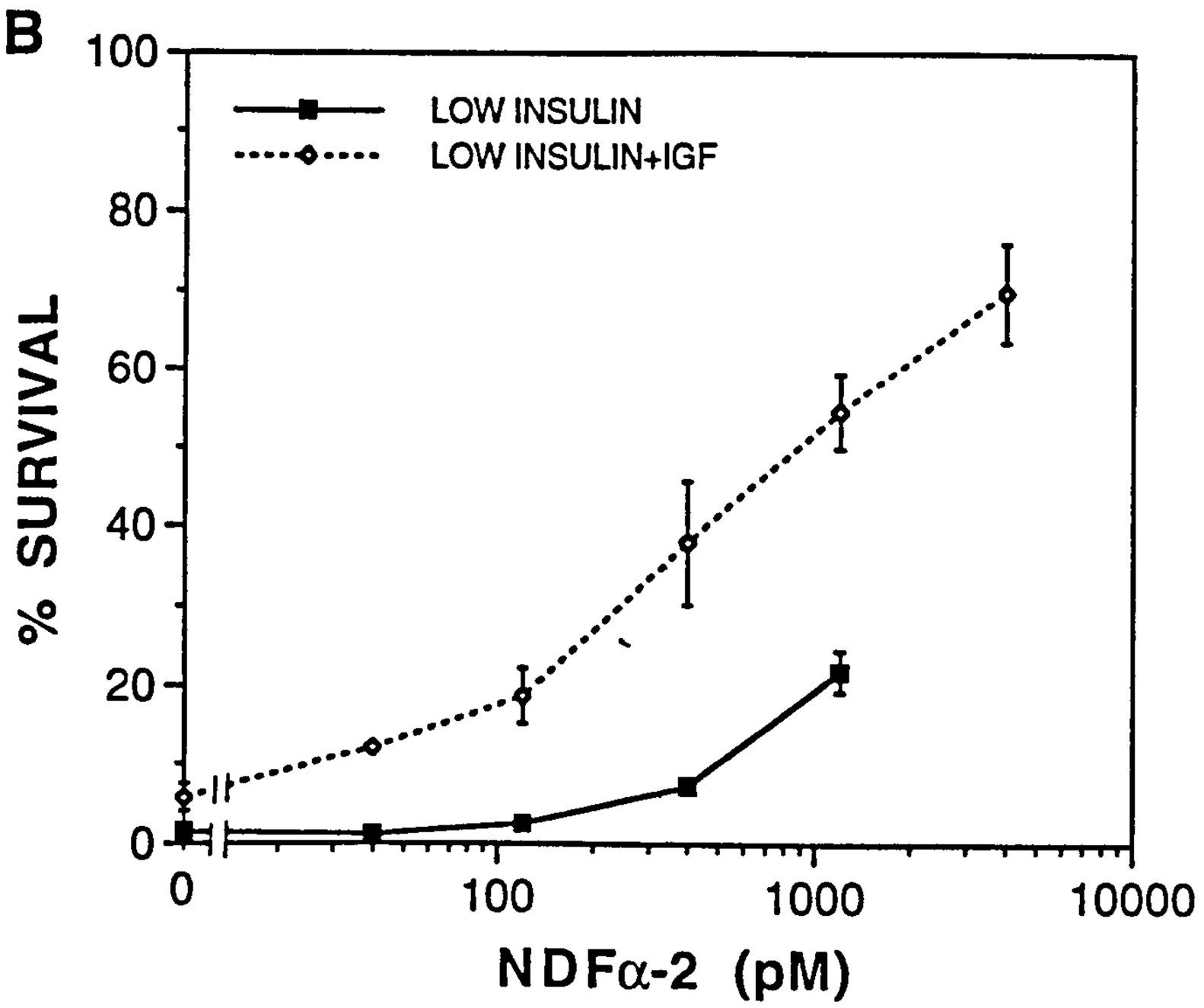
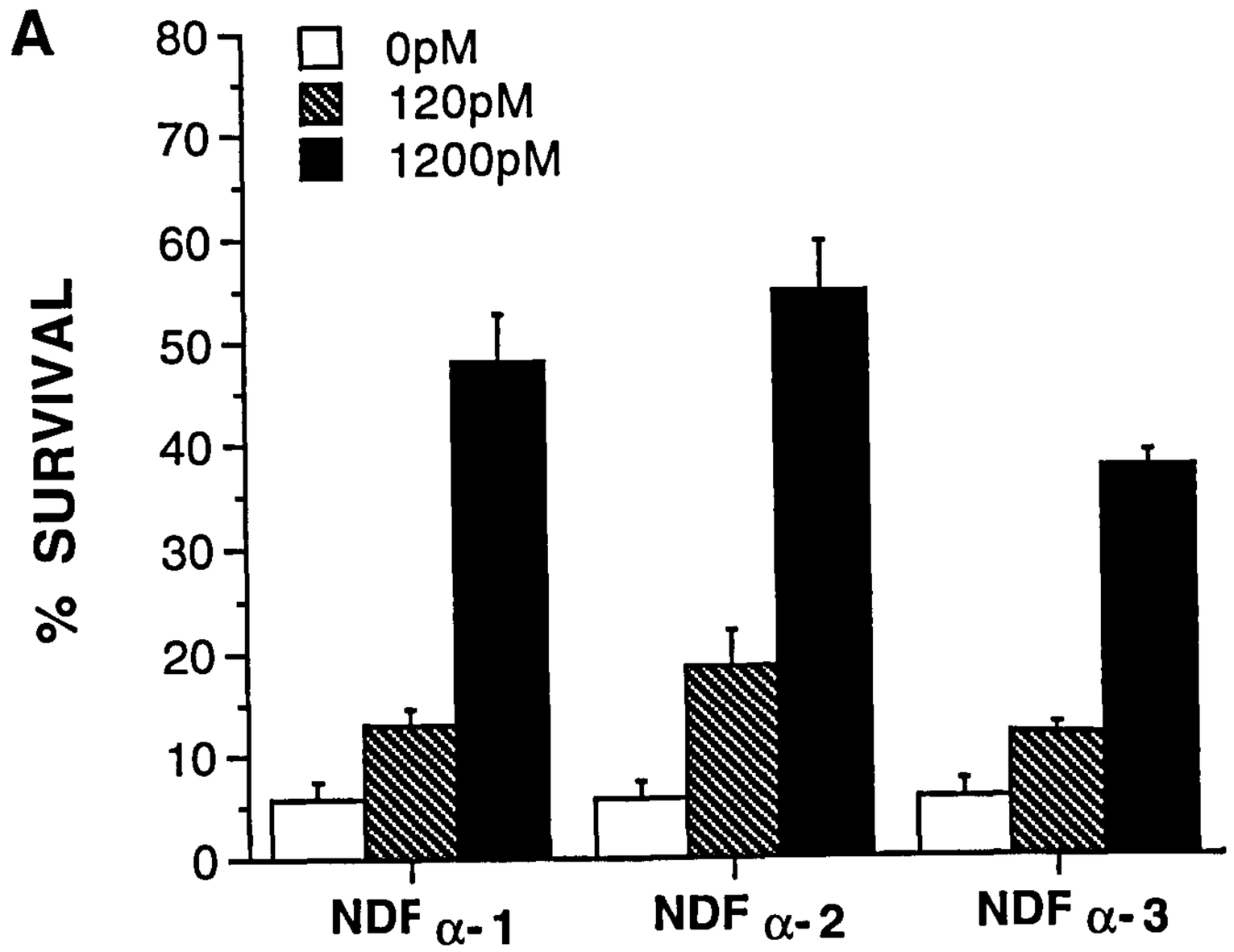

B
100
80
60
40
20
0
% SURVIVAL
LOW INSULIN
LOW INSULIN+IGF
0
100
1000
10000
NDFα-2 (pM)

Figure 3.5 NDFα isoforms share the same low survival activity for precursors

Schwann cell precursors were cultured in defined medium containing IGF-1 (13nM) plus different NDFα isoforms (A), or NDF-EGFα-2 domain (B). The result show that all NDFα isoforms share the same low survival activity and EGFα-2 domain alone has even less survival activity than that of NDFαs.


A
0pM
120pM
1200pM
% SURVIVAL
80
70
60
50
40
30
20
10
0
NDF α-1
NDF α-2
NDF α-3

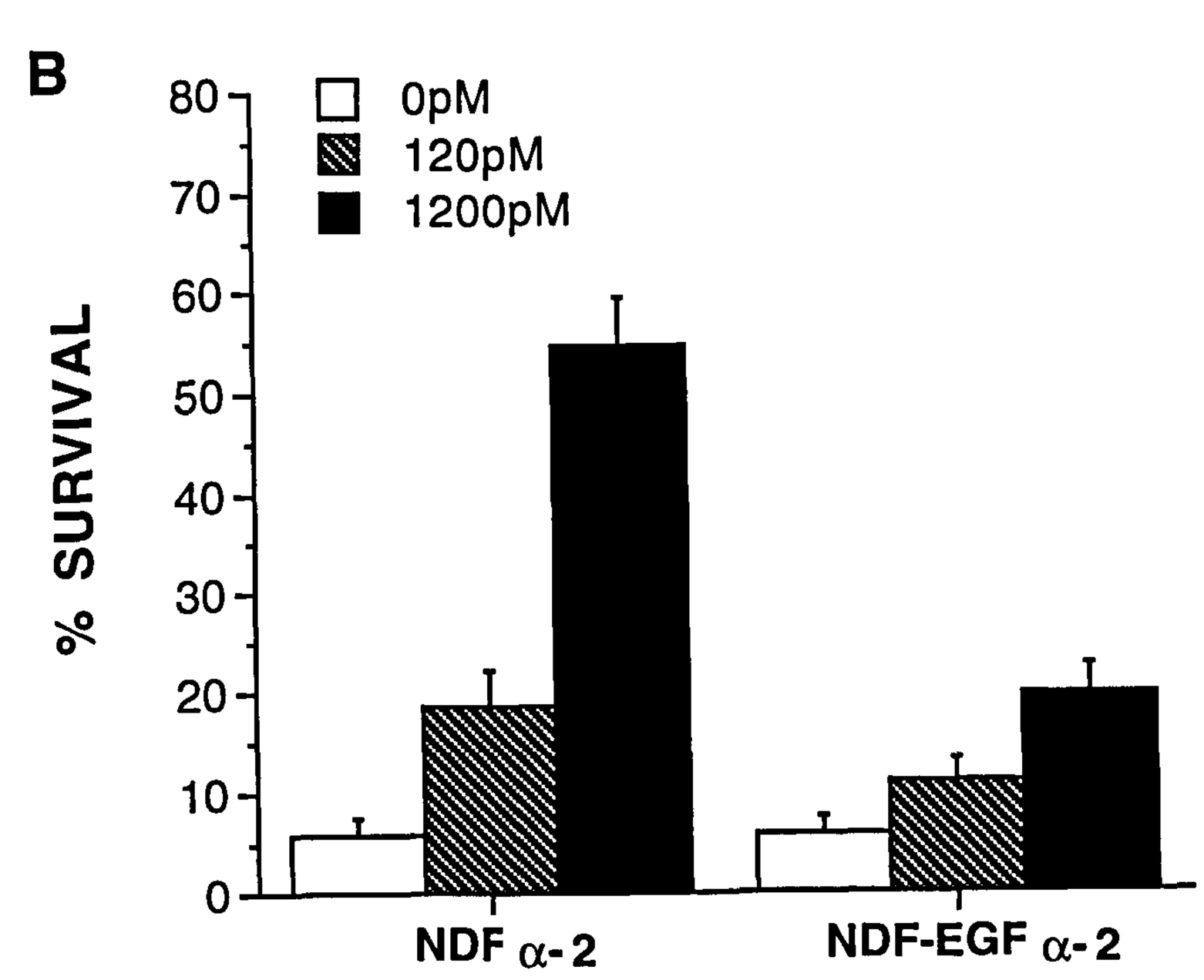
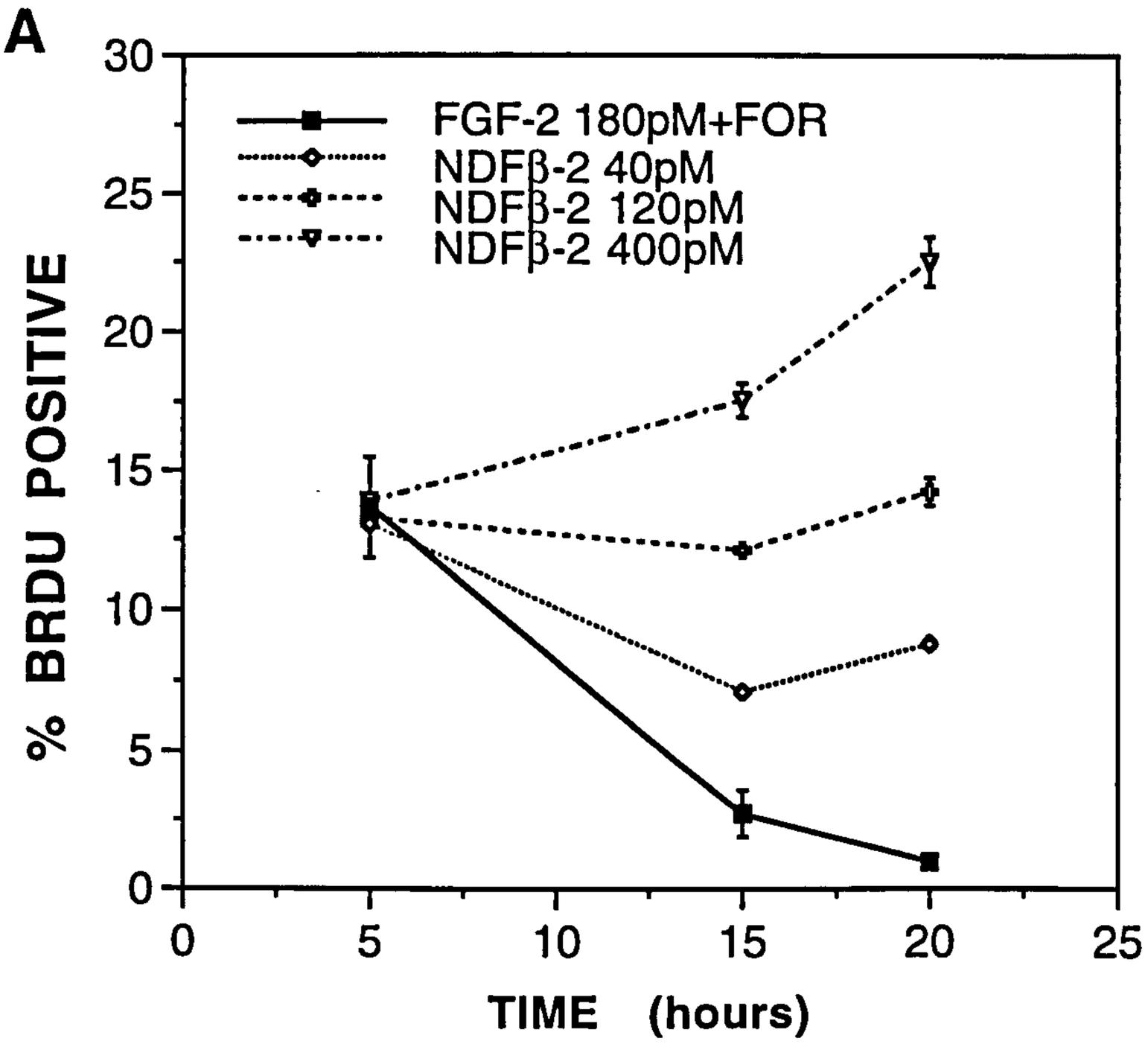

B
0pM
120pM
1200pM
% SURVIVAL
80
70
60
50
40
30
20
10
0
NDF α-2
NDF-EGF α-2

Figure 3.6 NDFβ-2 acts as a dose-dependent mitogen for precursors

(A) Schwann cell precursors were cultured in defined medium containing FGF-2 (180pM) plus forskolin (5μM) or NDFβ-2 in different concentrations. BrdU was introduced into the culture at the 3.5 hr, 13.5 hr and 18.5 hr points with 1.5 hr pulse each (B) Schwann cell precursors were cultured in FGF-2 plus forskolin for 20 hr then the cultured medium was changed to NDFβ-2 containing medium for a further 20 hr of culture. DNA synthesis was monitored at the 5 hr, 15 hr, 20 hr, 25 hr and 40 hr points with 1.5 hr BrdU pulse each. The results show that NDFβ-2 promotes DNA synthesis of precursors in dose-dependent manner.

A


FGF-2 180pM+FOR
NDFβ-2 40pM
NDFβ-2 120pM
NDFβ-2 400pM
% BRDU POSITIVE
0
5
10
15
20
25
TIME (hours)

B

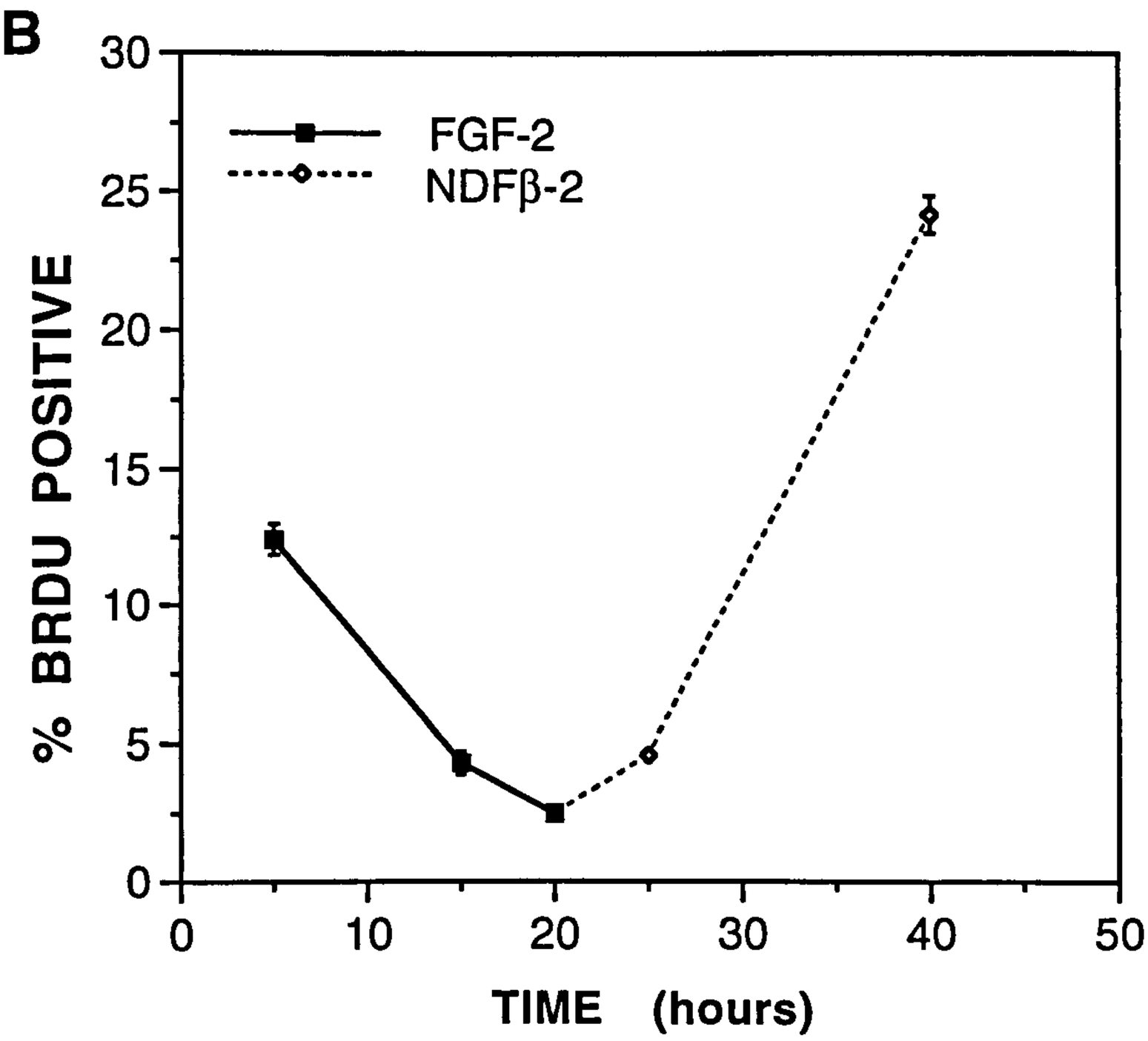
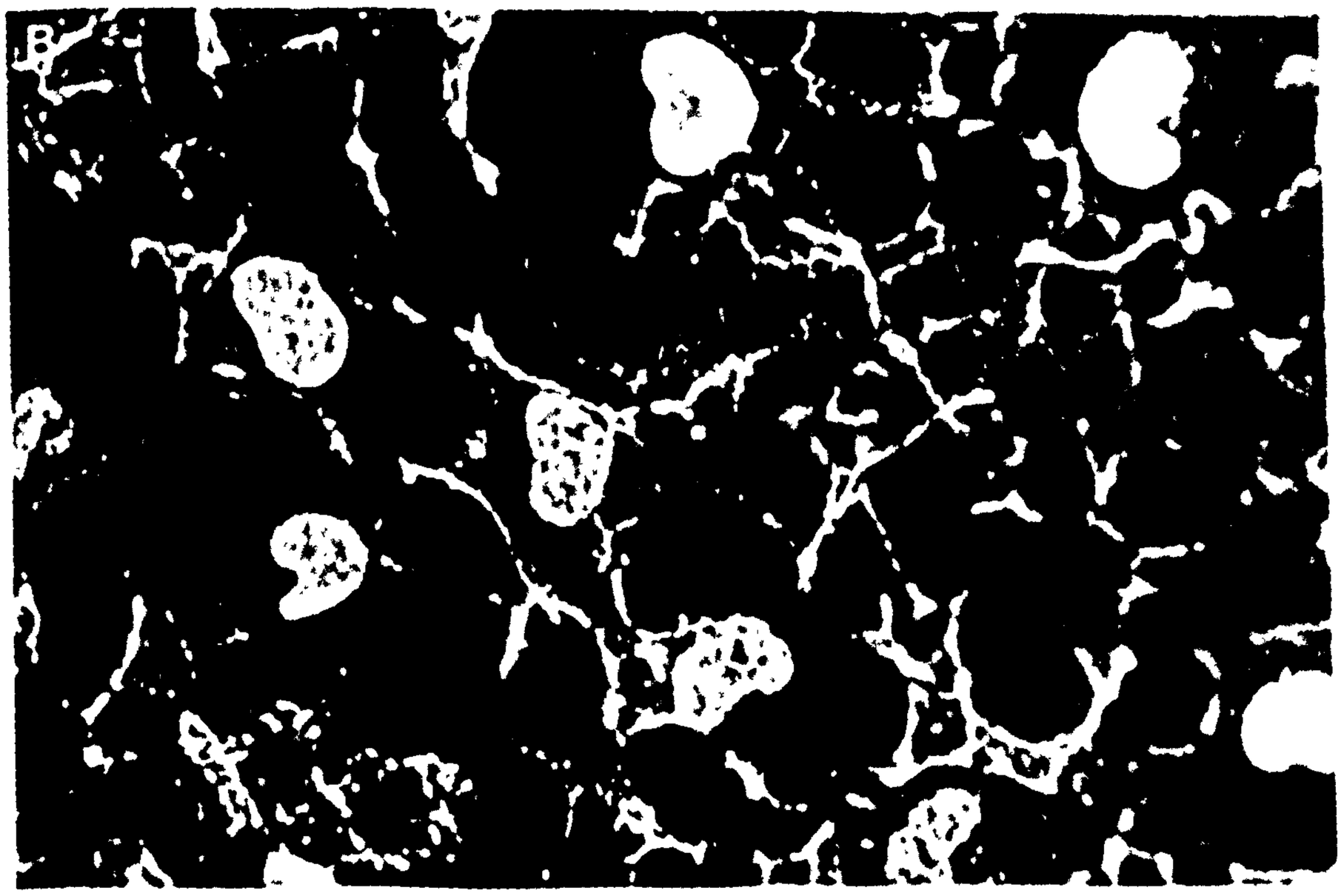

FGF-2
NDFβ-2
% BRDU POSITIVE
0
5
10
15
20
25
30
0
10
20
30
40
50
TIME (hours)

Figure 3.7 NDFβ stimulates DNA synthesis of Schwann cell precursors

Schwann cell precursors were cultured in NDFβ containing medium for 20 hr (A). 1.5 hr BrdU pulse was used during 18.5-20 hr. The precursors were double labeled with L1 (B) and BrdU (C). Magnification 800X


A


B

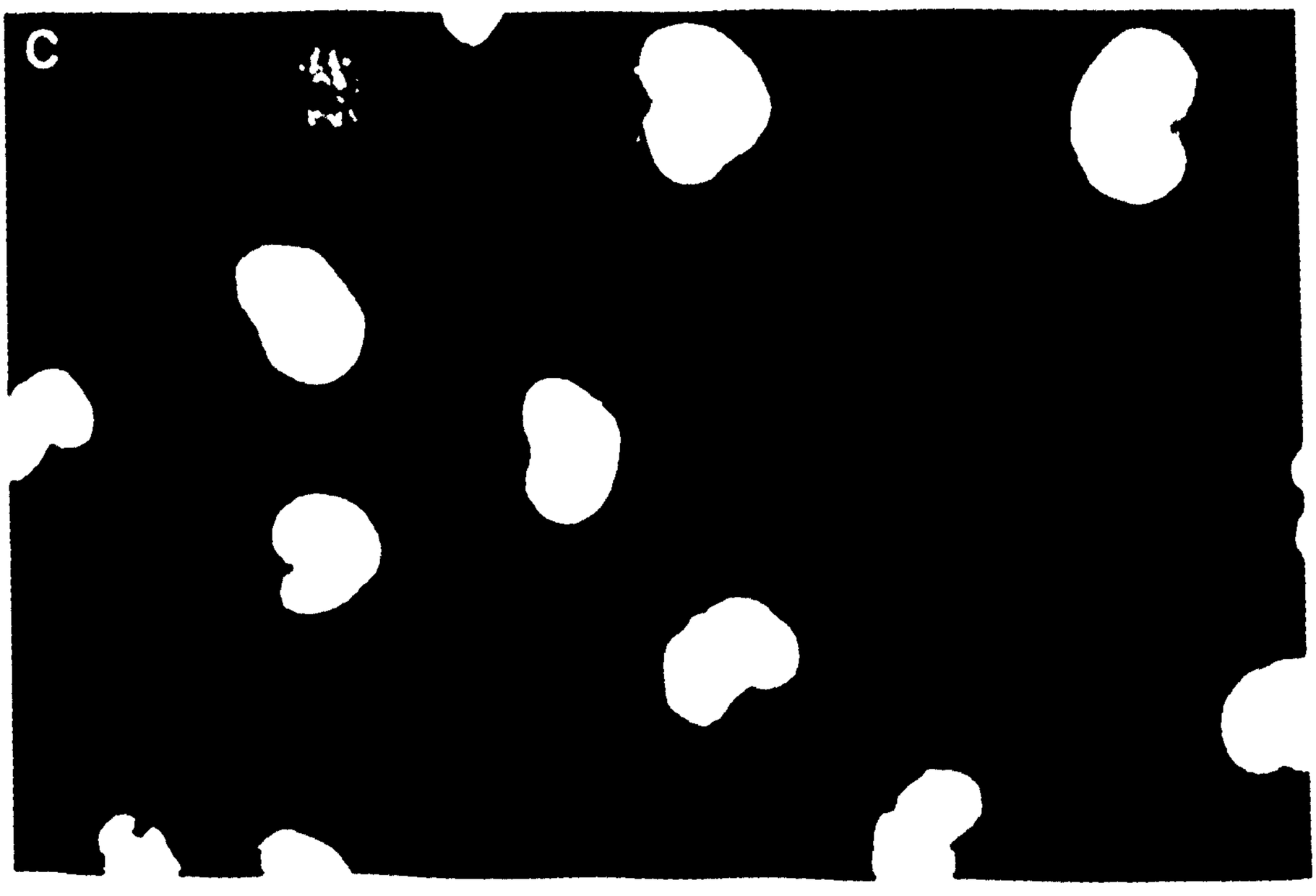
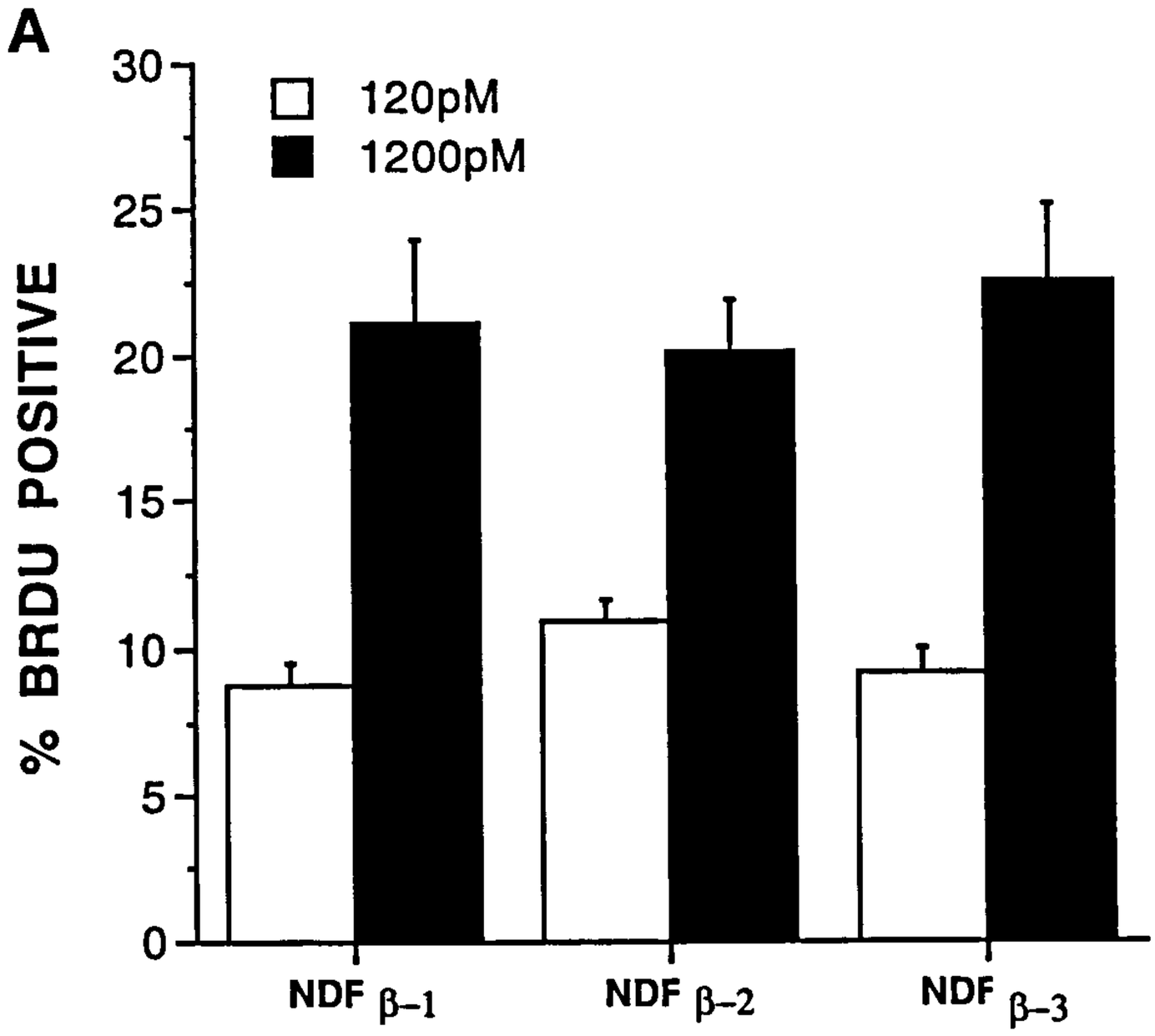

C

Figure 3.8 NDFβs share a similar mitogenic potential

Schwann cell precursors were cultured in defined medium containing different isoforms of NDFβ (A), and NDF-EGFβ-1 or NDF-EGFβ domain (B) for 20 hr with 1.5 hr BrdU pulse. The results show all NDFβ isoforms have the same mitogenic activity and EGFβ domain alone was able to trigger the DNA synthesis in precursors.


A
30
25
20
15
10
5
0
120pM
1200pM
% BRDU POSITIVE
NDF β–1
NDF β–2
NDF β–3

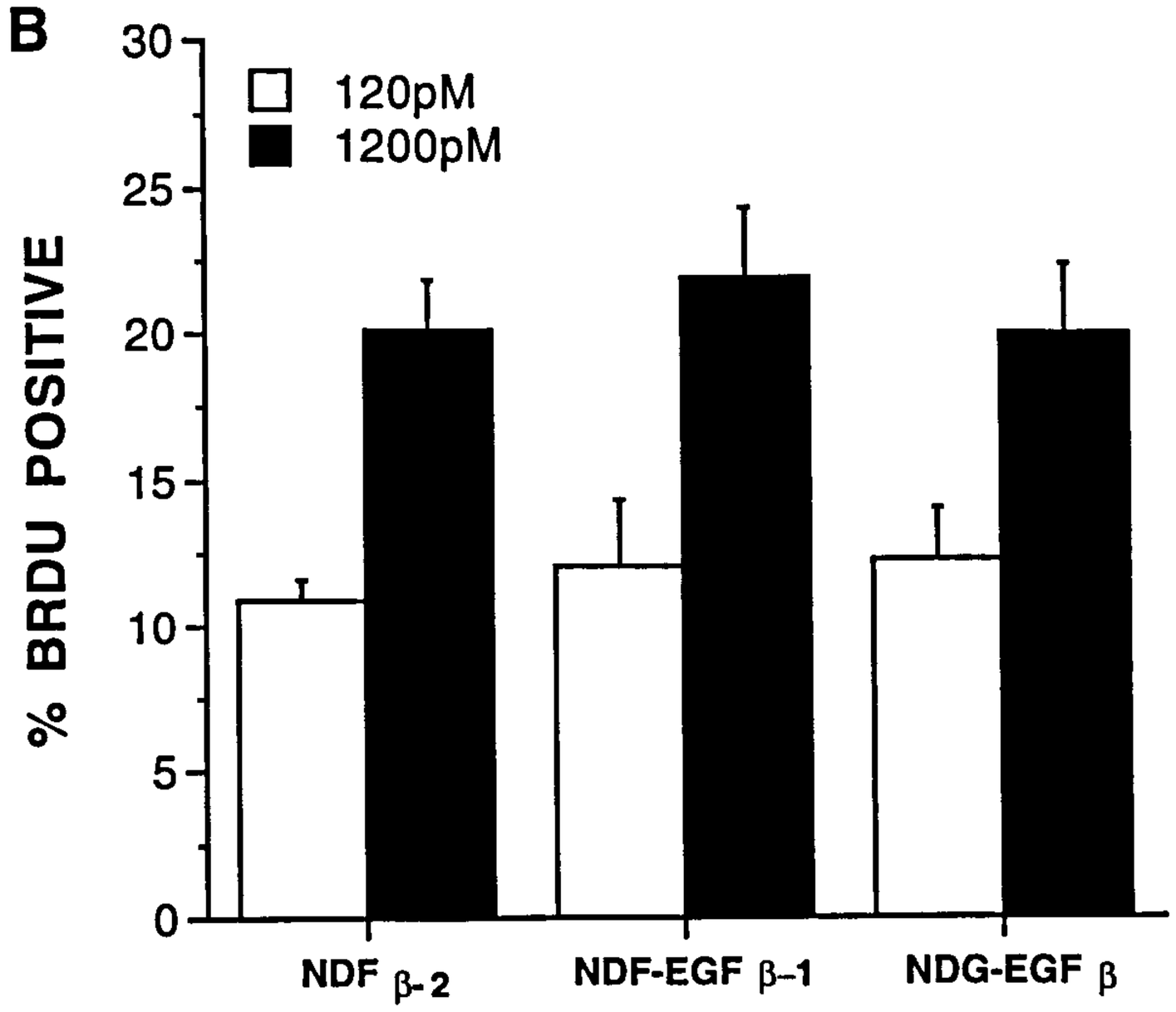

B
30
25
20
15
10
5
0
120pM
1200pM
% BRDU POSITIVE
NDF β-2
NDF-EGF β–1
NDG-EGF β

Figure 3.9 NDFβ retains mitogenic potential for precursors in low insulin medium

Schwann cell precursors were cultured in defined medium containing low insulin or low insulin plus IGF-1 (13nM). NDFβ-2 dose-response curves were constructed under these two conditions. BrdU was introduced into culture for the last 1.5 hr of total 20 hr assay. The results show that NDFβ also stimulates DNA synthesis of precursors in the absence of IGF.

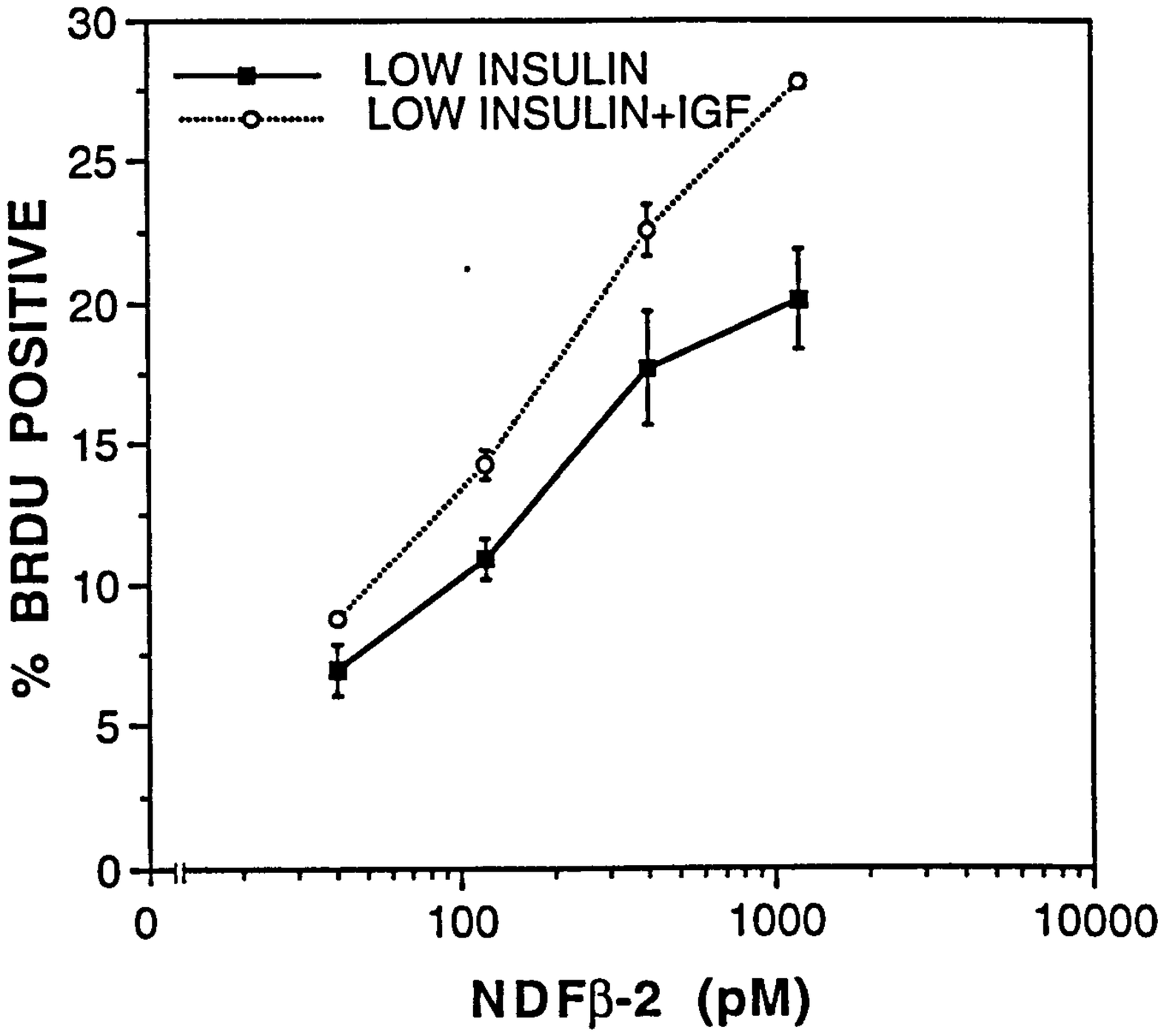

LOW INSULIN
LOW INSULIN+IGF
% BRDU POSITIVE
30
25
20
15
10
5
0
0
100
1000
10000
NDFβ-2 (pM)

Figure 3.10 TGFβ acts as mitogen for precursors in the presence of FIF medium

(A) Schwann cell precursors were cultured FIF medium (FGF-2 180pM plus IGF-1 13nM and forskolin 5μM) or FIF medium plus TGFβ1 (1ng/ml) for 20 hr, 1.5 pulse BrdU was used at the 18.5 hr point. (B) Schwann cell precursors were cultured in FIF medium plus various concentrations of TGFβ1 for a total of 44 hr, and a 20 hr BrdU pulse was used at the 24 hr point. The results showed that TGFβ in the presence of FGF and forskolin stimulates DNA synthesis in Schwann cell precursors and acts in a dose-dependent manner.

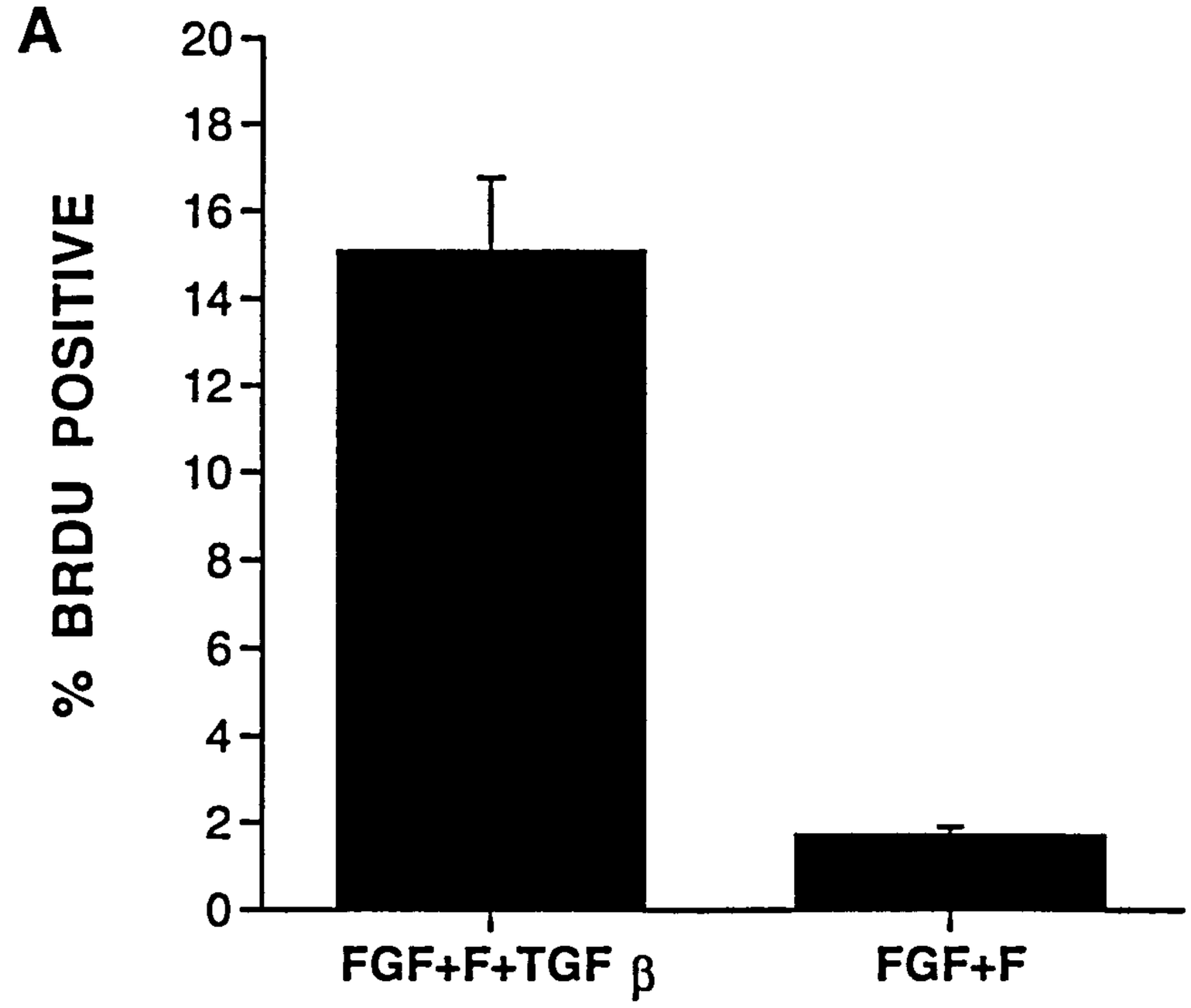
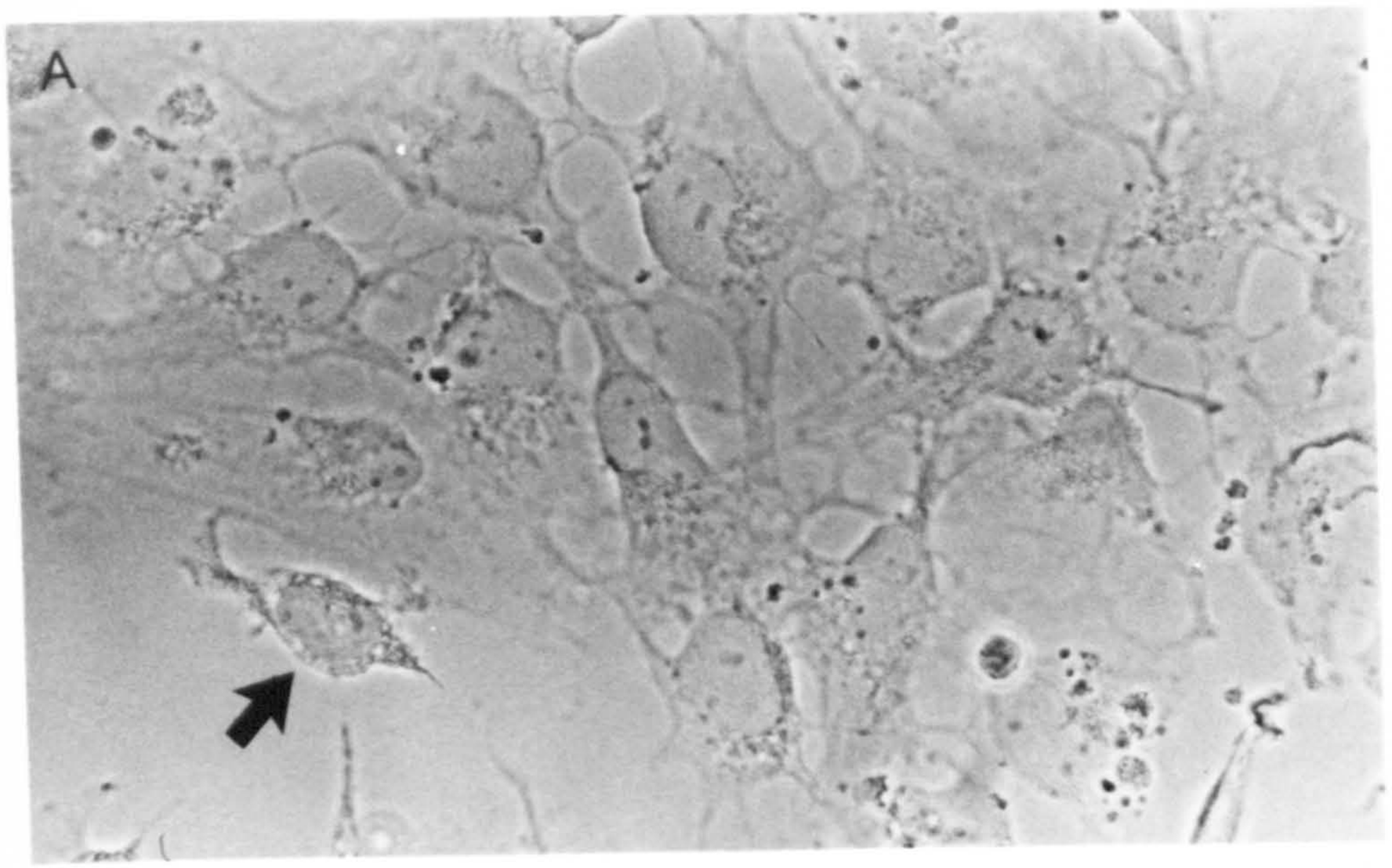
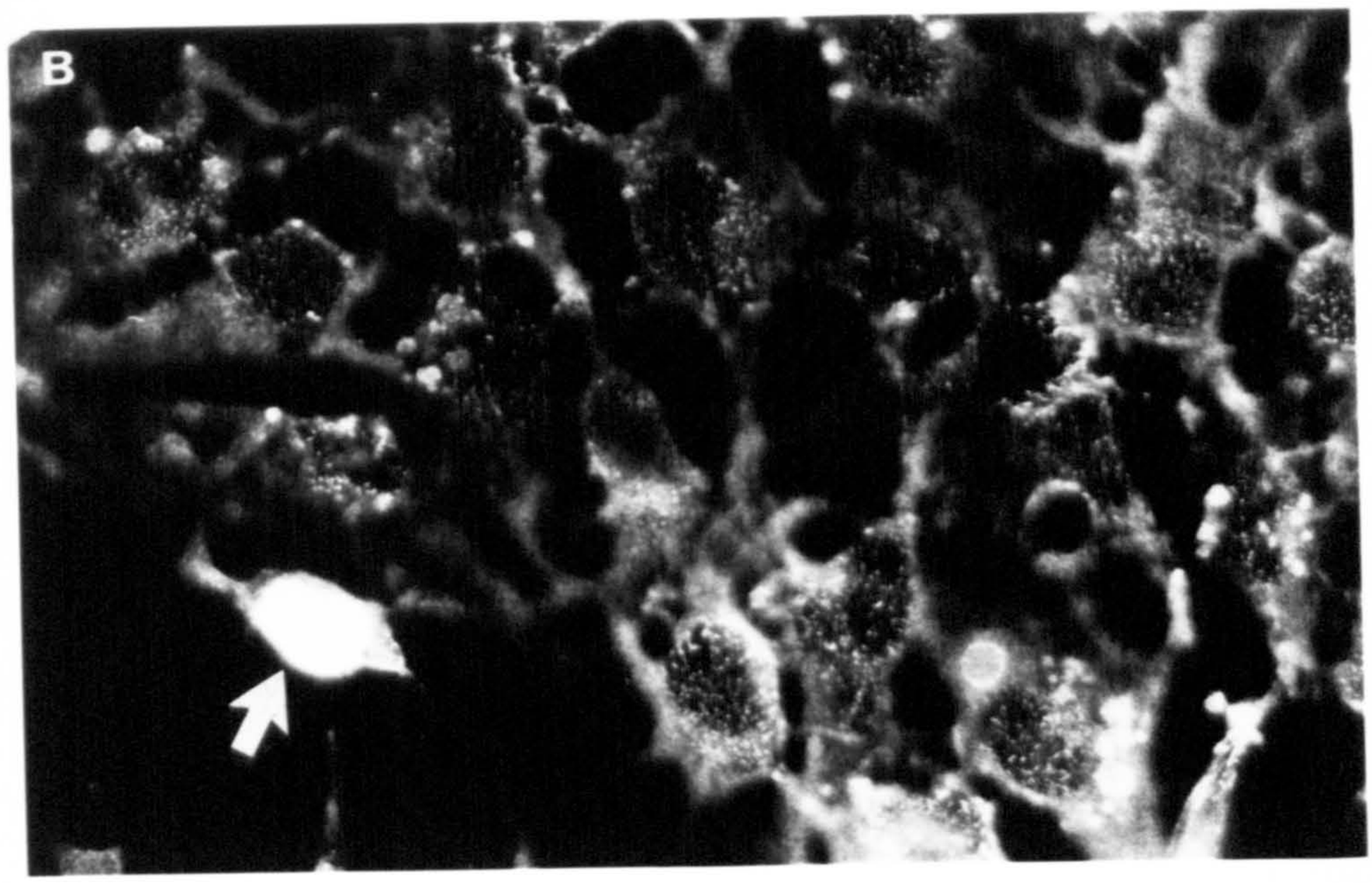

A
% BRDU POSITIVE
20
18
16
14
12
10
8
6
4
2
0
FGF+F+TGF β
FGF+F
B
% BRDU POSITIVE
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
TGFβ-1 (ng/ml)

Figure 3.11 $L1^-/p75NGF\text{-}R^+$ cells dissociated from E14 nerves respond to FGF plus forskolin

The cells dissociated from E14 nerves were cultured in defined medium containing FGF-2 (180pM) plus forskolin (5μM) for 20 hr. 1.5 hr BrdU pulse was used. L1, p75NGF-R and BrdU triple-labelling was applied. (A) phase-contrast view of (B) and (C). (B) p75NGF-R and BrdU staining. (C) L1 labelling. The results show that the isolated bipolar cells (arrows) are L1 negative and p75NGF-R positive and BrdU positive, while group-forming cells are generally L1 positive, p75NGF-R positive and BrdU negative (A,B,C). Magnification 800X


A


B

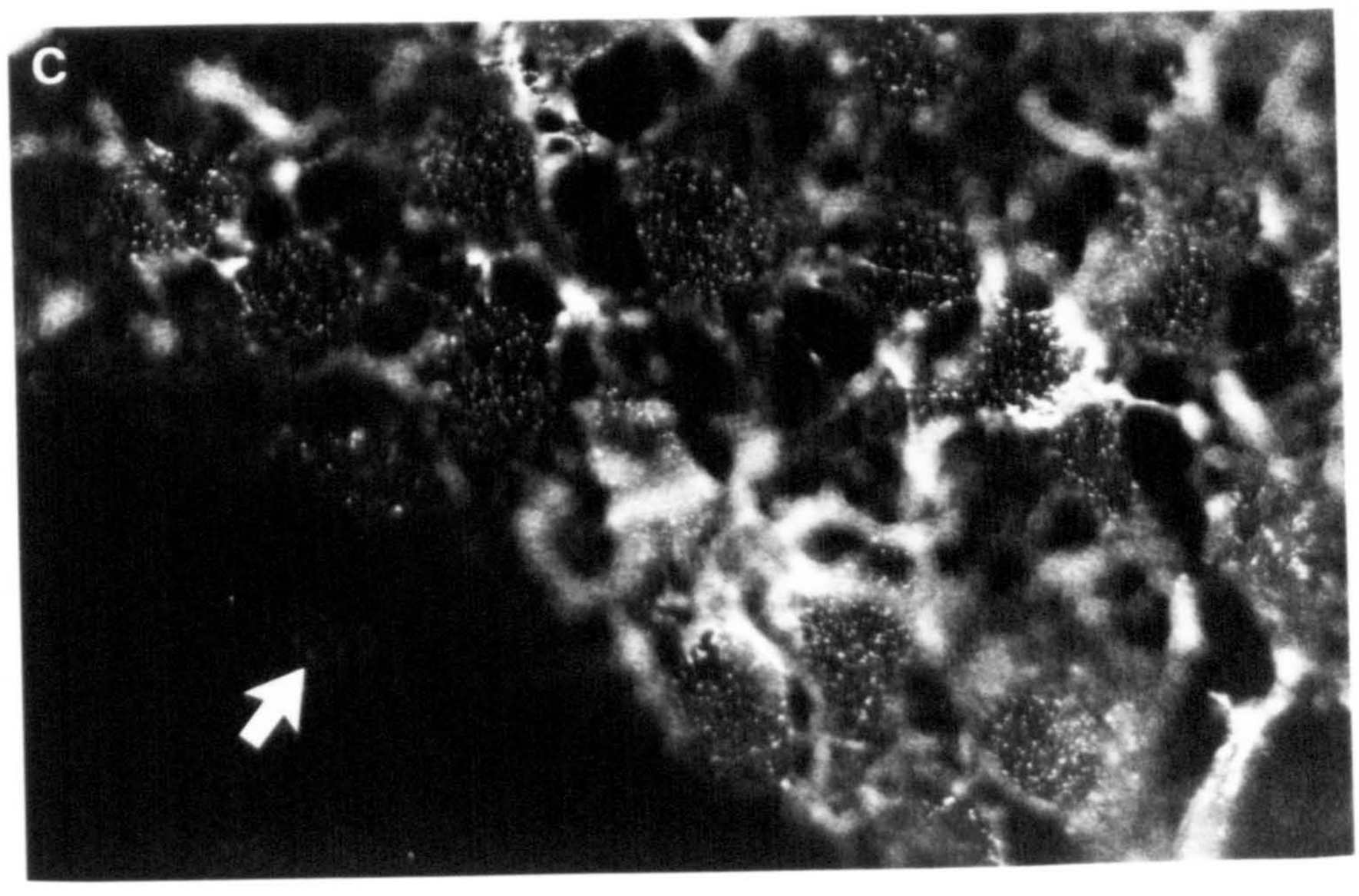
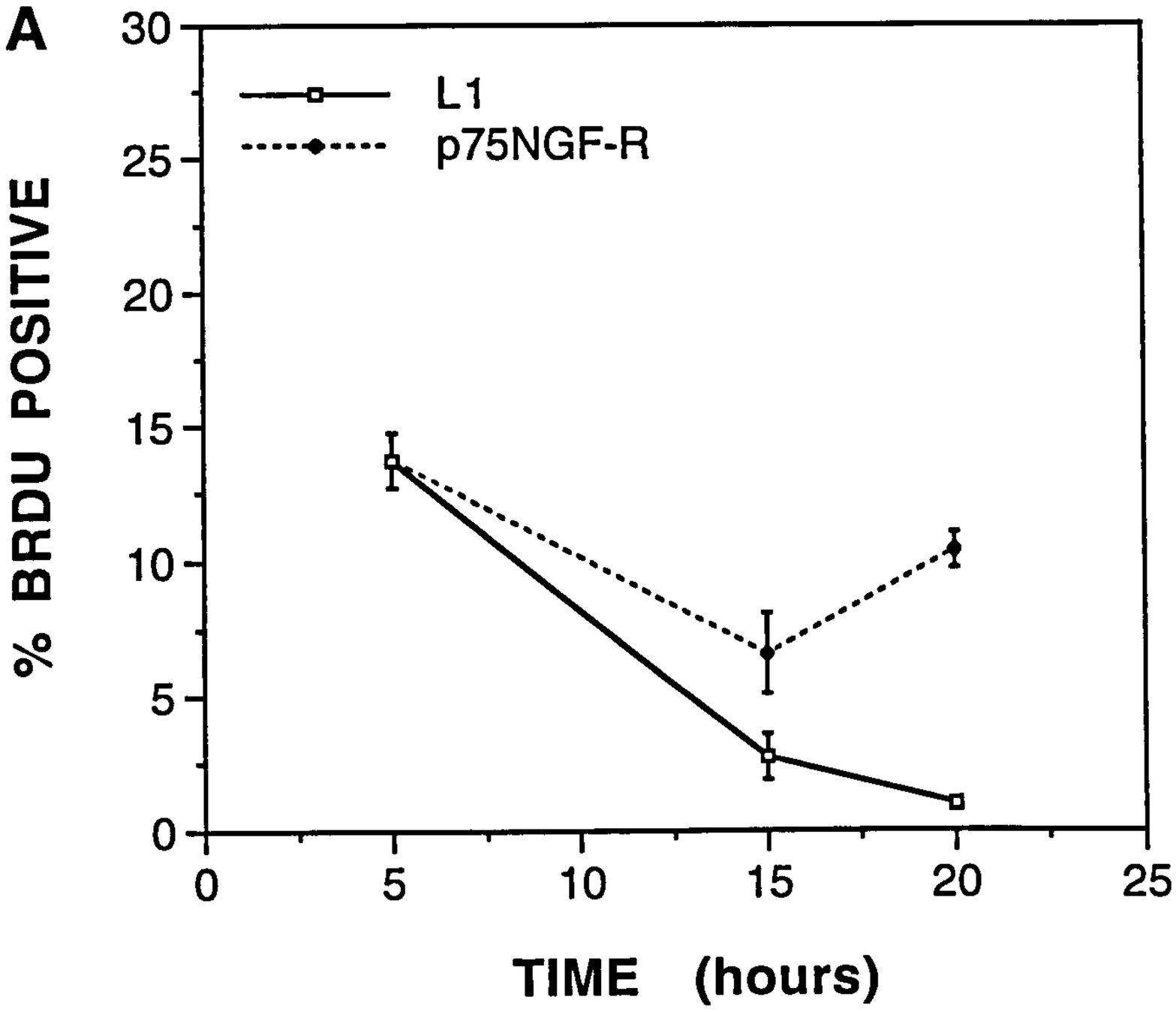

C

Figure 3.12 $L1^+$/p75NGF-R^+ cells and $L1^-$/p75NGF-R^+ cells respond differently to FGF-2 plus forskolin, but in the same manner to NDFβ-2

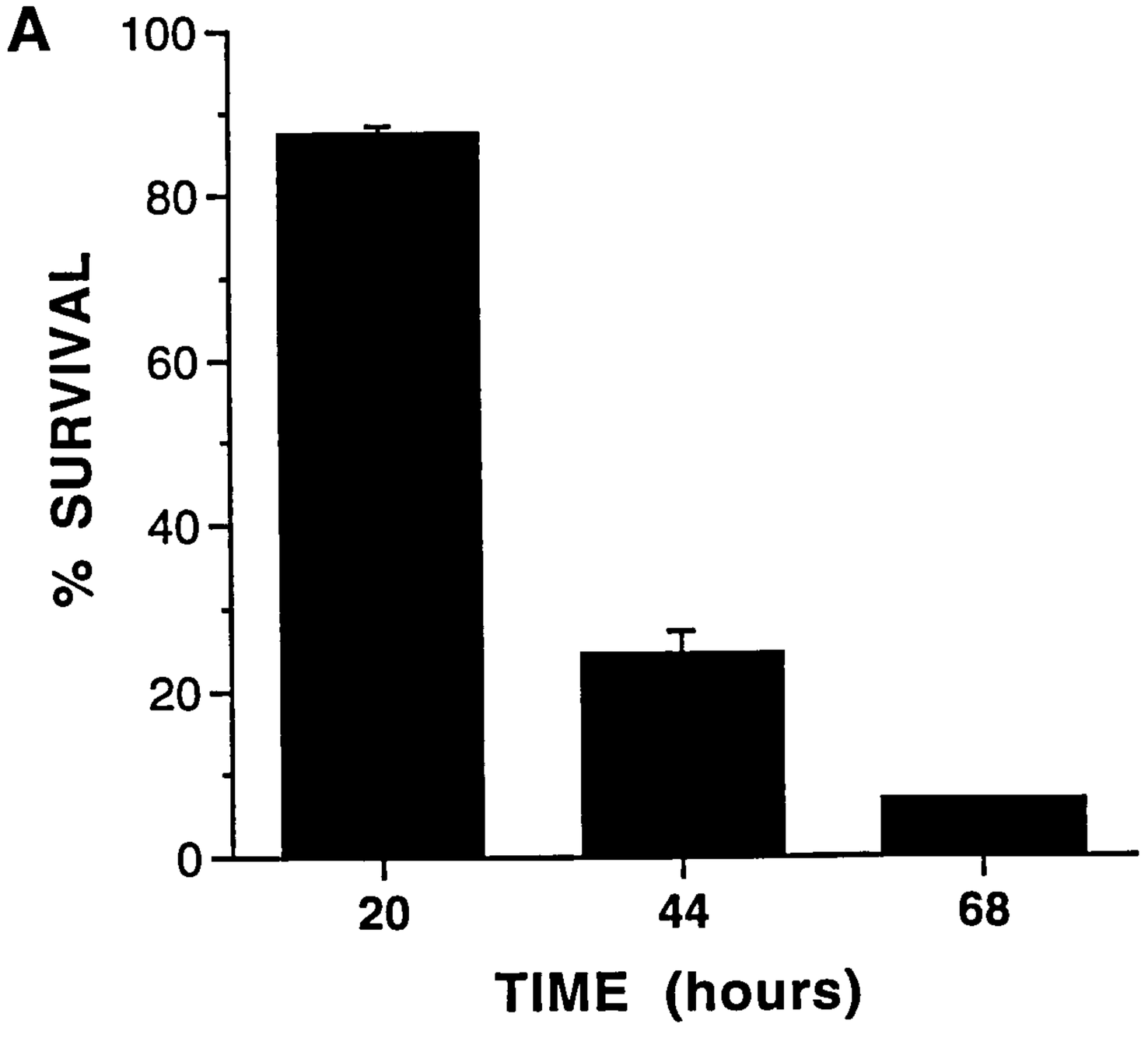
Schwann cell precursors were cultured in defined medium containing (A) FGF-2 (180pM) plus forskolin (5μM) and IGF-1 (13nM), (B) NDFβ-2 (400pM) plus IGF-1 (13nM) for 20 hr. L1, p75 NGF-R and BrdU triple-labelling was used for these precursors after 20 hr in culture. The results show that FGF-2 plus forskolin was mitogenic for p75NGF-R^+/$L1^-$ cells but not for $L1^+$/p75NGF-R^+ cells. Whereas, NDFβ plus IGF was mitogenic for both cell types.


A
% BRDU POSITIVE
L1
p75NGF-R
0
5
10
15
20
25
30
TIME (hours)

B
% BRDU POSITIVE
L1
p75NGF-R
0
5
10
15
20
25
30
TIME (hours)

Figure 3.13 The long-term survival factor for Schwann cell precursors

Schwann cell precursors were cultured in defined medium containing (A) FGF-2 (180pM) plus IGF-1 (13nM) or (B) NDFβ-2 (32pM) plus IGF-1 (13nM) for 20 hr, 44 hr and 68 hr. The culture medium was changed at the 20 hr and the 44 hr points. L1 antibodies were used to label the surviving precursors. The results show that NDFβ supports precursor survival for more than 68 hr, while FGF-2 only supports precursor survival for 20 hr.


A
100
80
60
40
20
0
% SURVIVAL
20
44
68
TIME (hours)

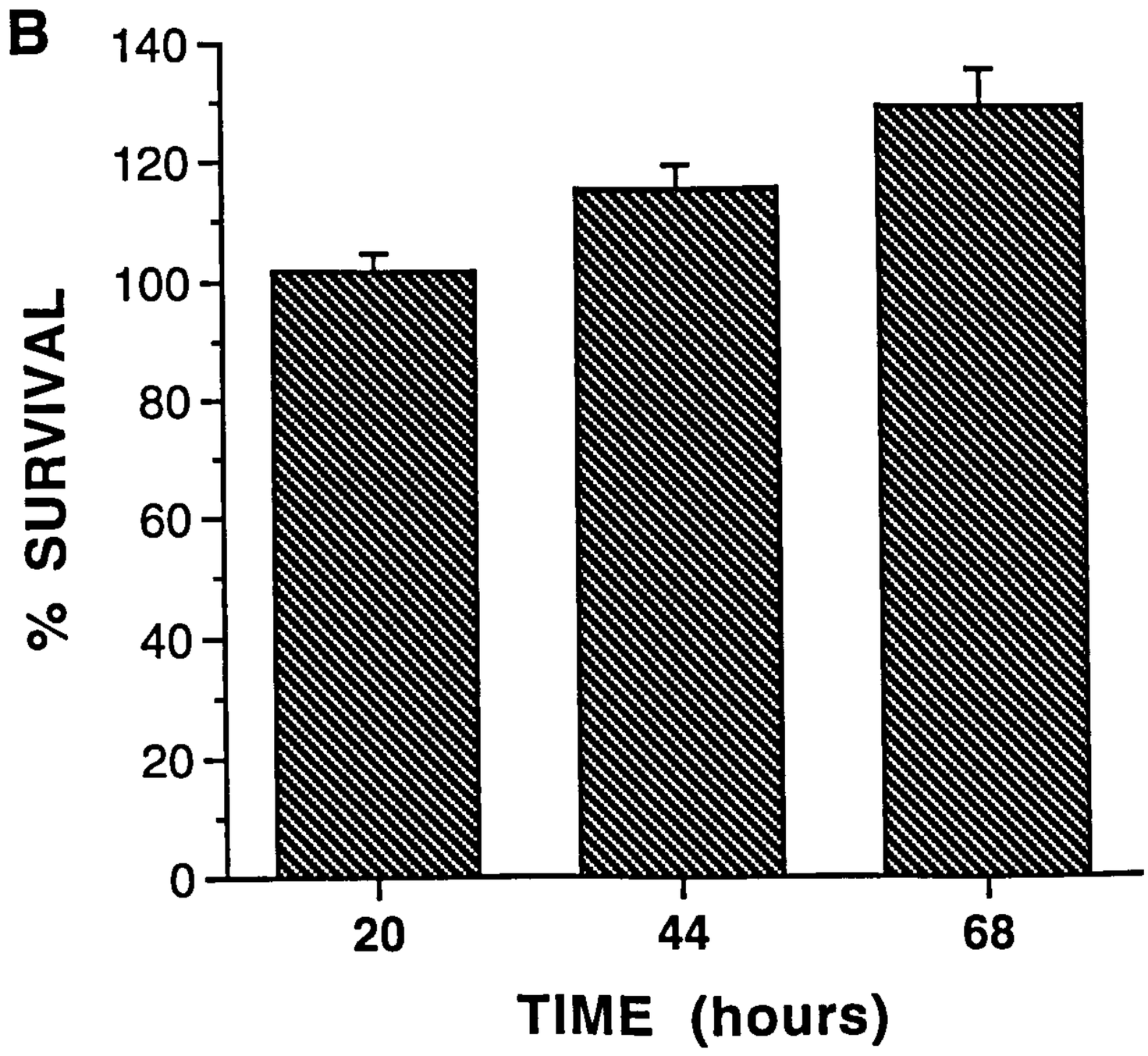
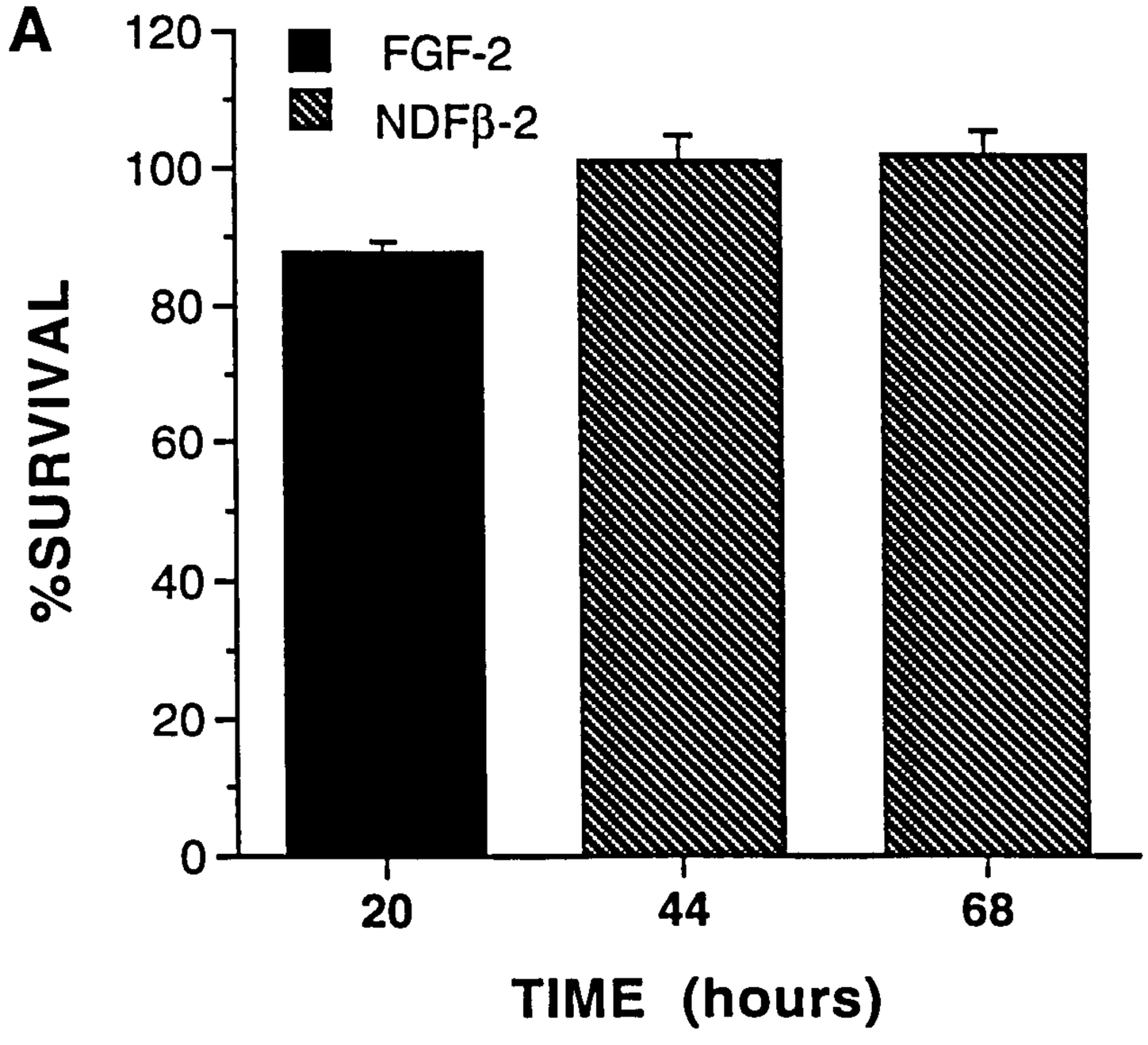

B
140
120
100
80
60
40
20
0
% SURVIVAL
20
44
68
TIME (hours)

Figure 3.14 FGF-2 maintains responsiveness of precursors to NDF

Schwann cell precursors were cultured in defined medium containing FGF-2 (180pM) plus IGF-1 (13nM) for 20 hr, then cultured precursors were washed several times with defined medium and changed to defined medium containing (A) NDFβ-2 (400pM) plus IGF-1 (13nM) or (B) NDFα-2 (400pM) plus IGF-1 (13nM) for a further 24 hr or 48 hr. The total L1 positive cells at the 20 hr, 44 hr and 68 hr points were counted. The results show that NDFβ supports survival of all precursors after these cells are cultured in FGF-2 for 20 hr, whereas, NDFα only rescues about 25% of precursors.


A
120
100
80
60
40
20
0
%SURVIVAL
FGF-2
NDFβ-2
20
44
68
TIME (hours)

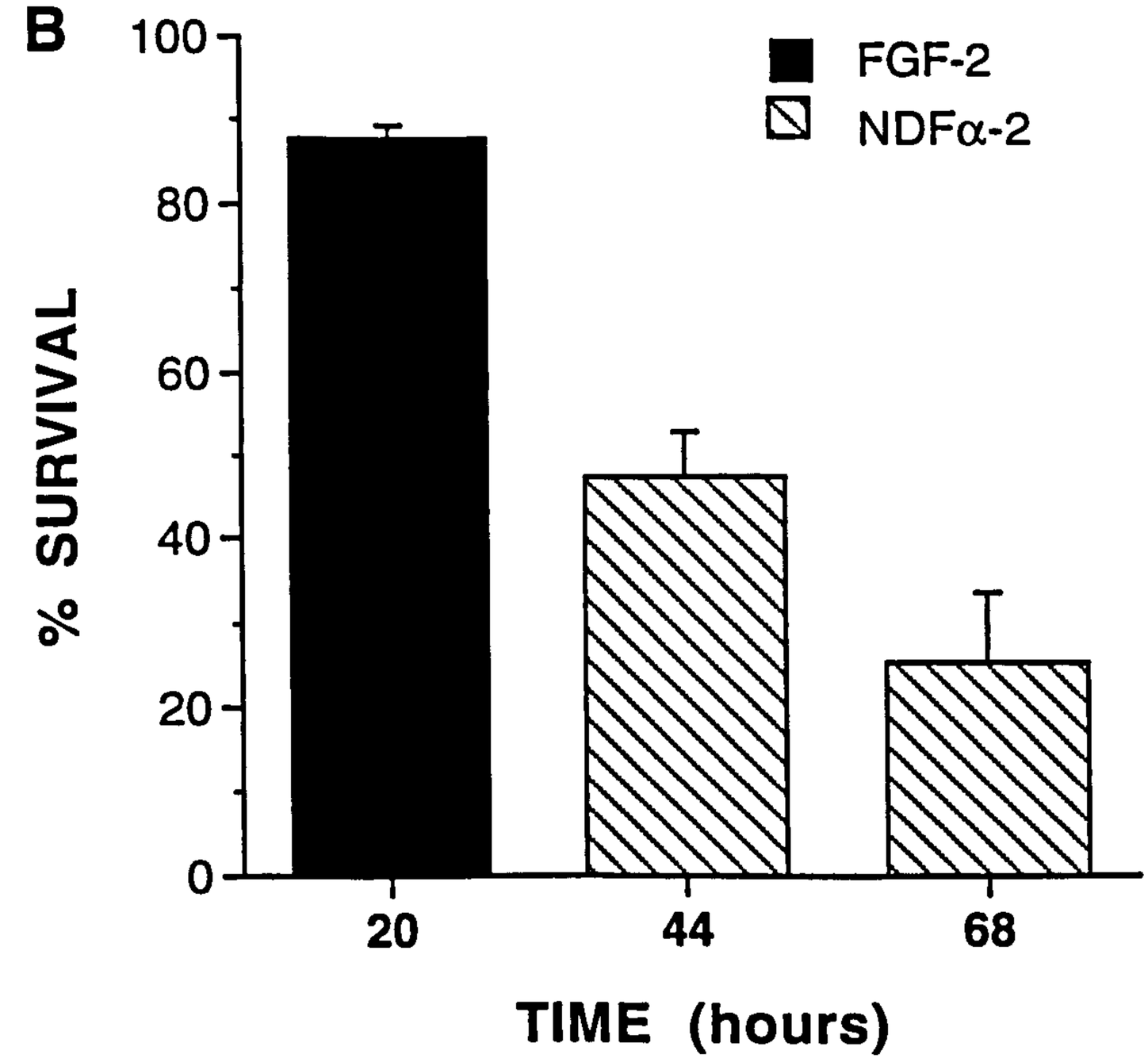
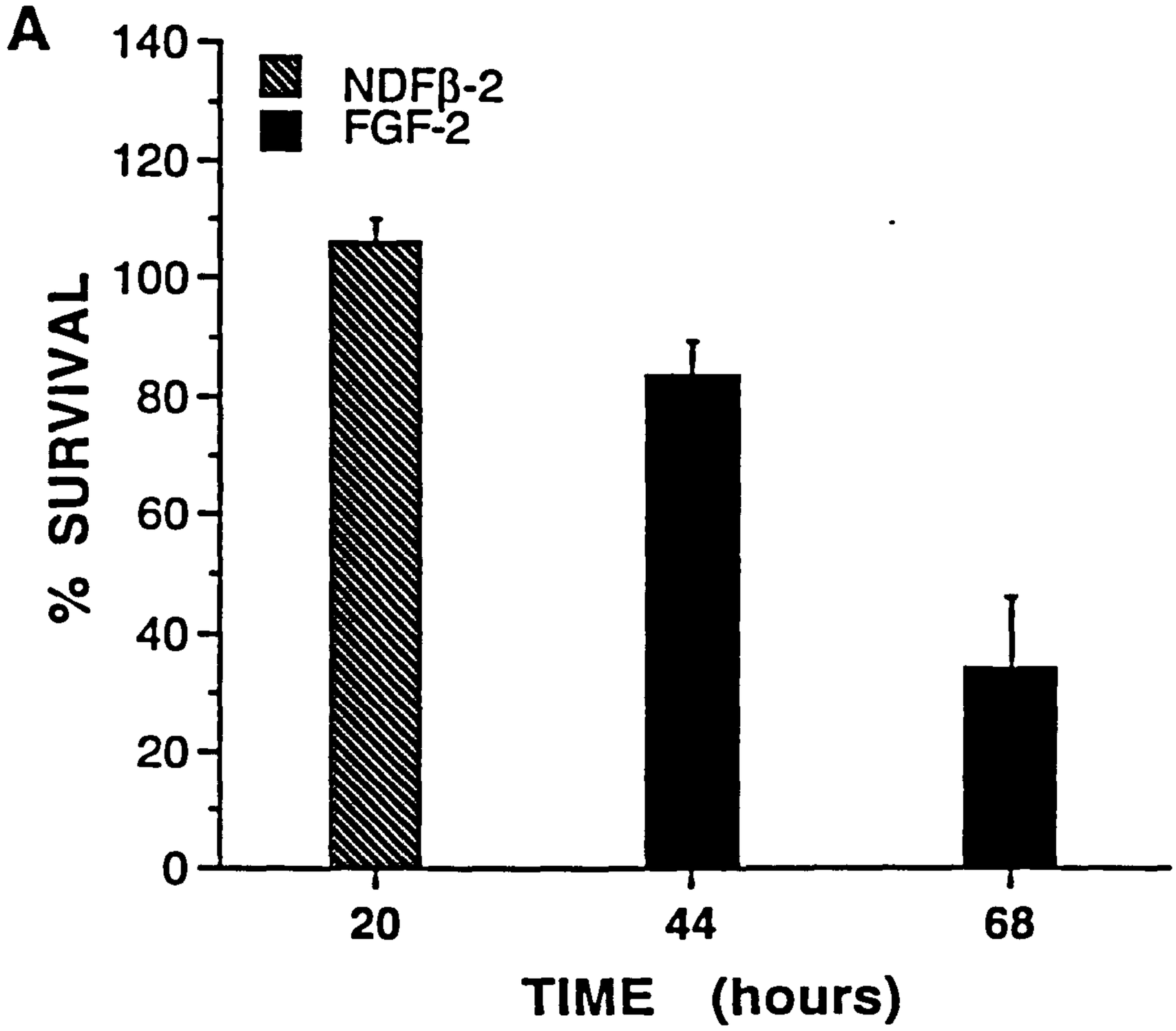

B
100
80
60
40
20
0
% SURVIVAL
FGF-2
NDFα-2
20
44
68
TIME (hours)

Figure 3.15 NDFβ maintains precursor responsiveness to FGF-2

Schwann cell precursors were cultured in defined medium containing NDFβ-2 (32nM) plus IGF-1 (13nM) for 1 day (A) or 2 days (B) then changed to FGF-2 (180pM) plus IGF-1 (13nM) containing medium. The surviving precursors were monitored at the 20 hr, 44 hr and 68 hr points as described before. The results show that NDFβ fully maintains responsiveness of precursors to FGF-2.


A
140
120
100
80
60
40
20
0
% SURVIVAL
NDFβ-2
FGF-2
20
44
68
TIME (hours)

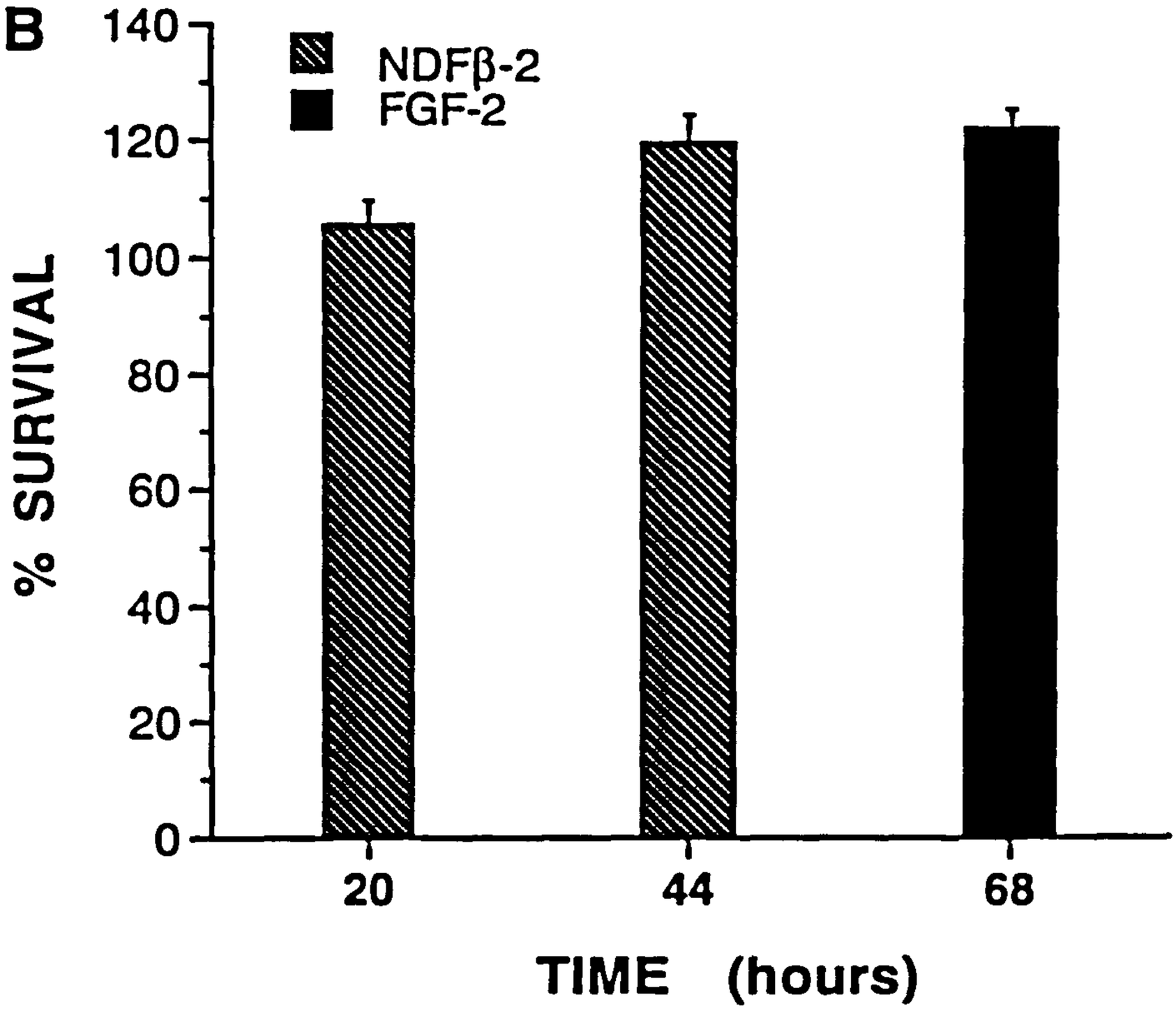
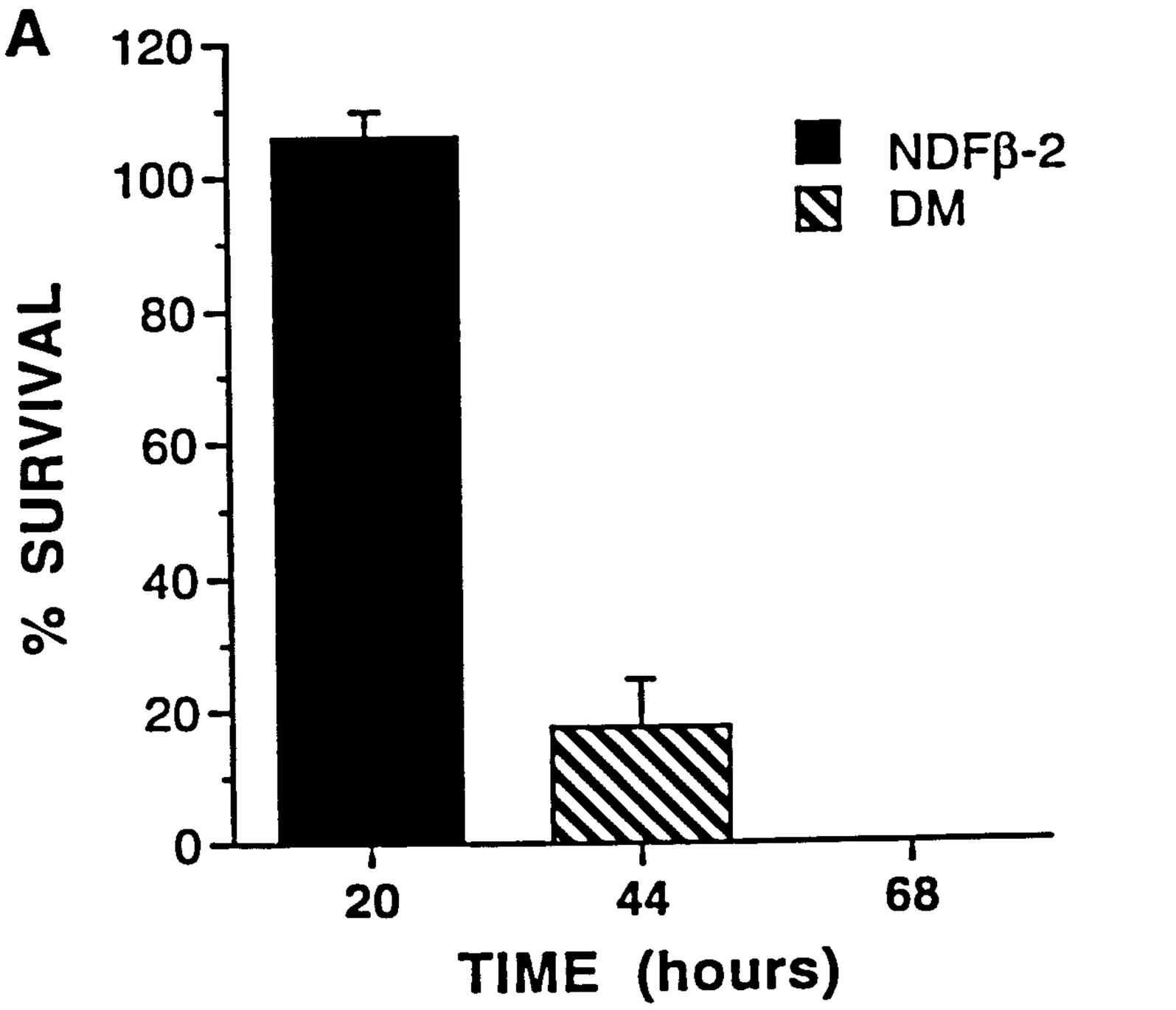

B
140
120
100
80
60
40
20
0
% SURVIVAL
NDFβ-2
FGF-2
20
44
68
TIME (hours)

Figure 3.16 NDFβ drives Schawnn cell precursor maturation

Schwann cell precursors were cultured in defined medium containing NDFβ-2 (32nM) plus IGF-1 (13nM) for 1 day (A) or 2 days (B). After washing the cultured cells were changed to defined medium only. The surviving precursors were monitored at the 20 hr, 44 hr and 68 hr points by L1 labelling. The results show that 50% of the L1 positive cells survive in defined medium after culturing in NDFβ-2 containing medium for 2 days.


A
120
100
80
60
40
20
0
% SURVIVAL
20
44
68
TIME (hours)
NDFβ-2
DM

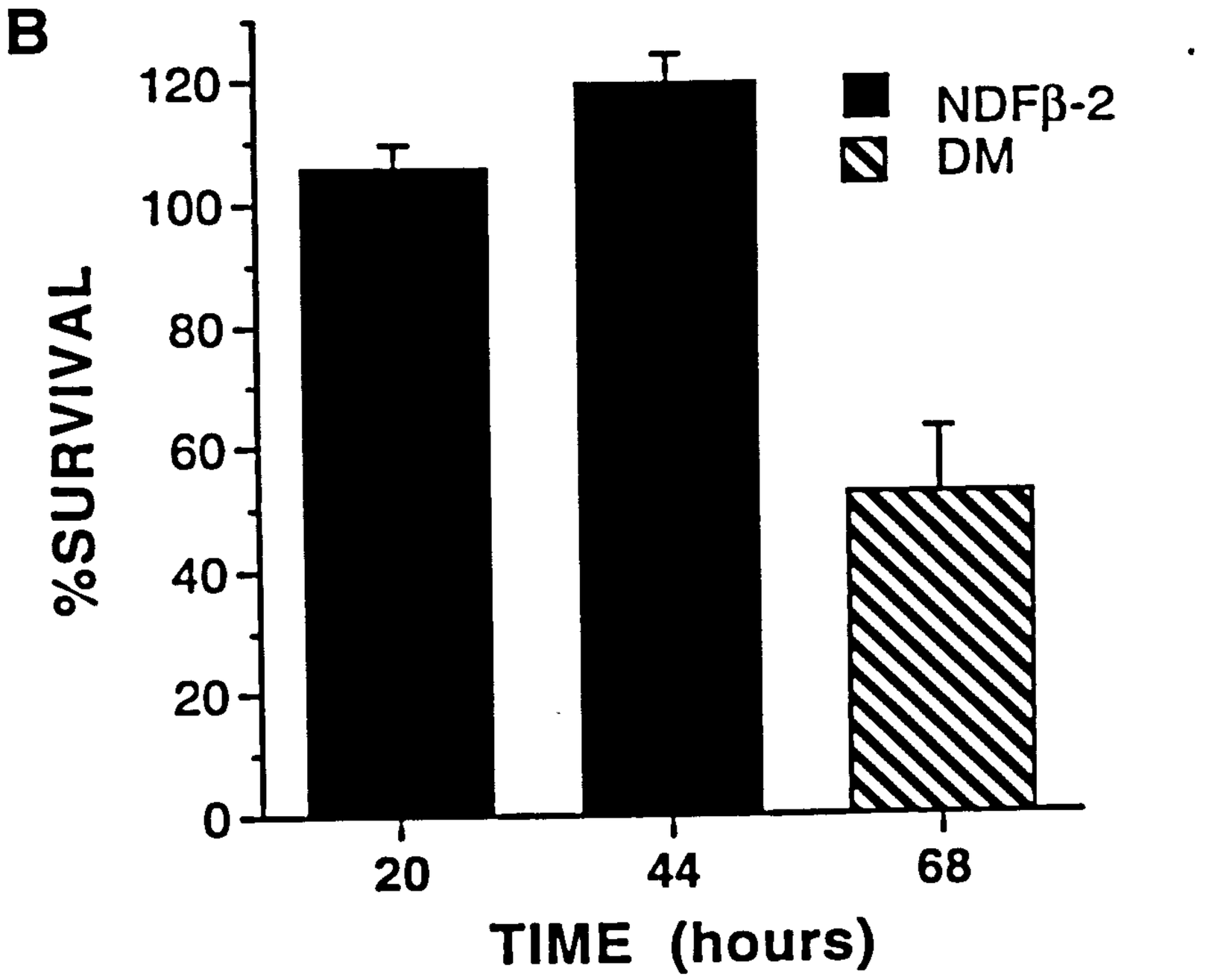

B
120
100
80
60
40
20
0
%SURVIVAL
20
44
68
TIME (hours)
NDFβ-2
DM

Figure 3.17 Anti NDF antibodies block the survival activity of NDF

(A) Various concentrations of polyclonal anti NDF antibodies 1915#, and (B) various concentrations of monoclonal antibodies 114A were mixed with NDFβ-2 (40pM) before introduction to the culture. Schwann cell precursors were plated in defined medium containing IGF-1 (13nM) for 3 hr, the cultured cells were then topped up with the antibodies containing medium for total 20 hr. The surviving precursors were labelled with L1 antibody. The results show that 1915# blocks 80% of the survival activity of NDFβ-2 (A), while 114A only diminishes the survival activity of NDFβ-2 to 48% (B).

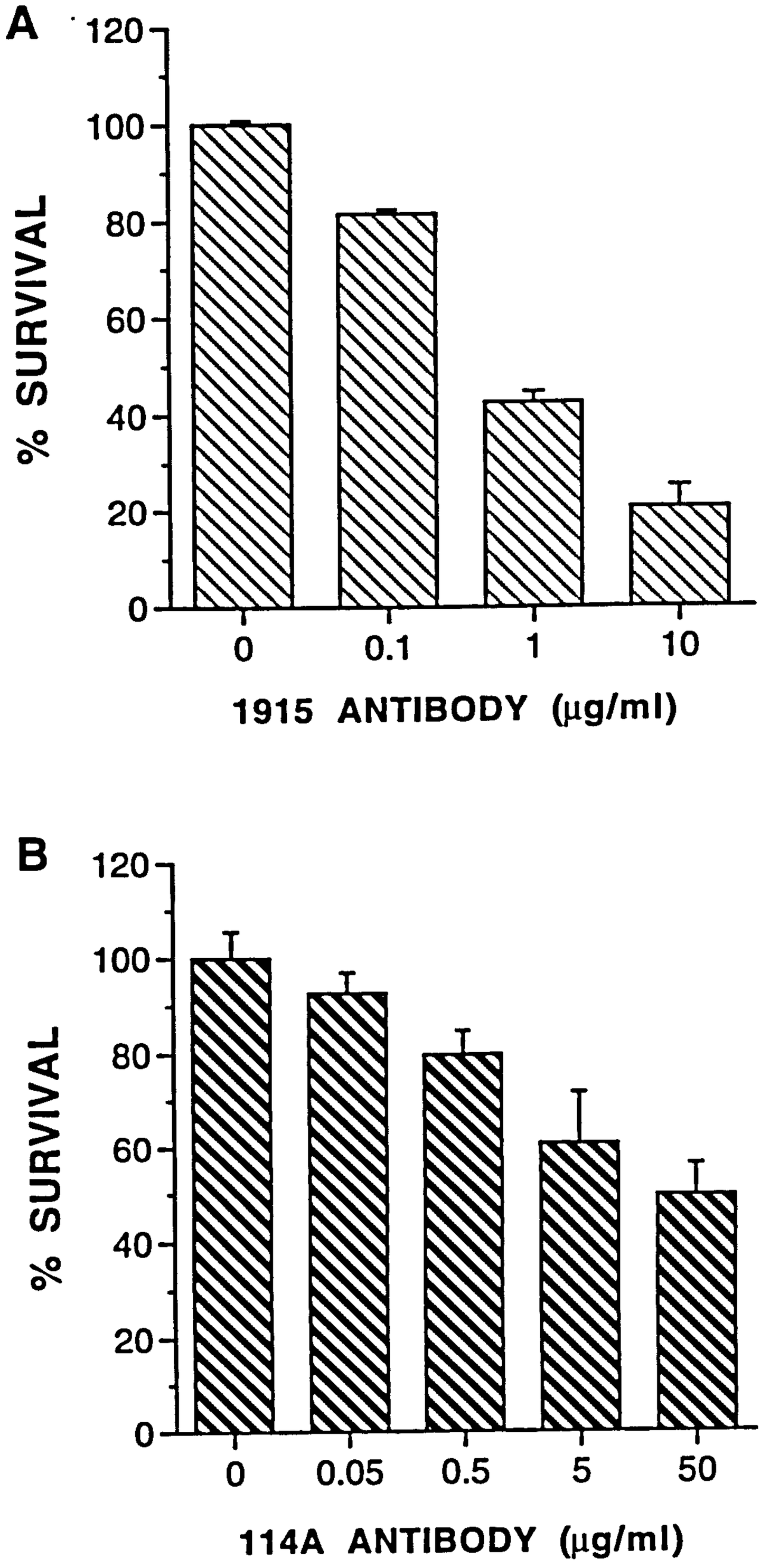
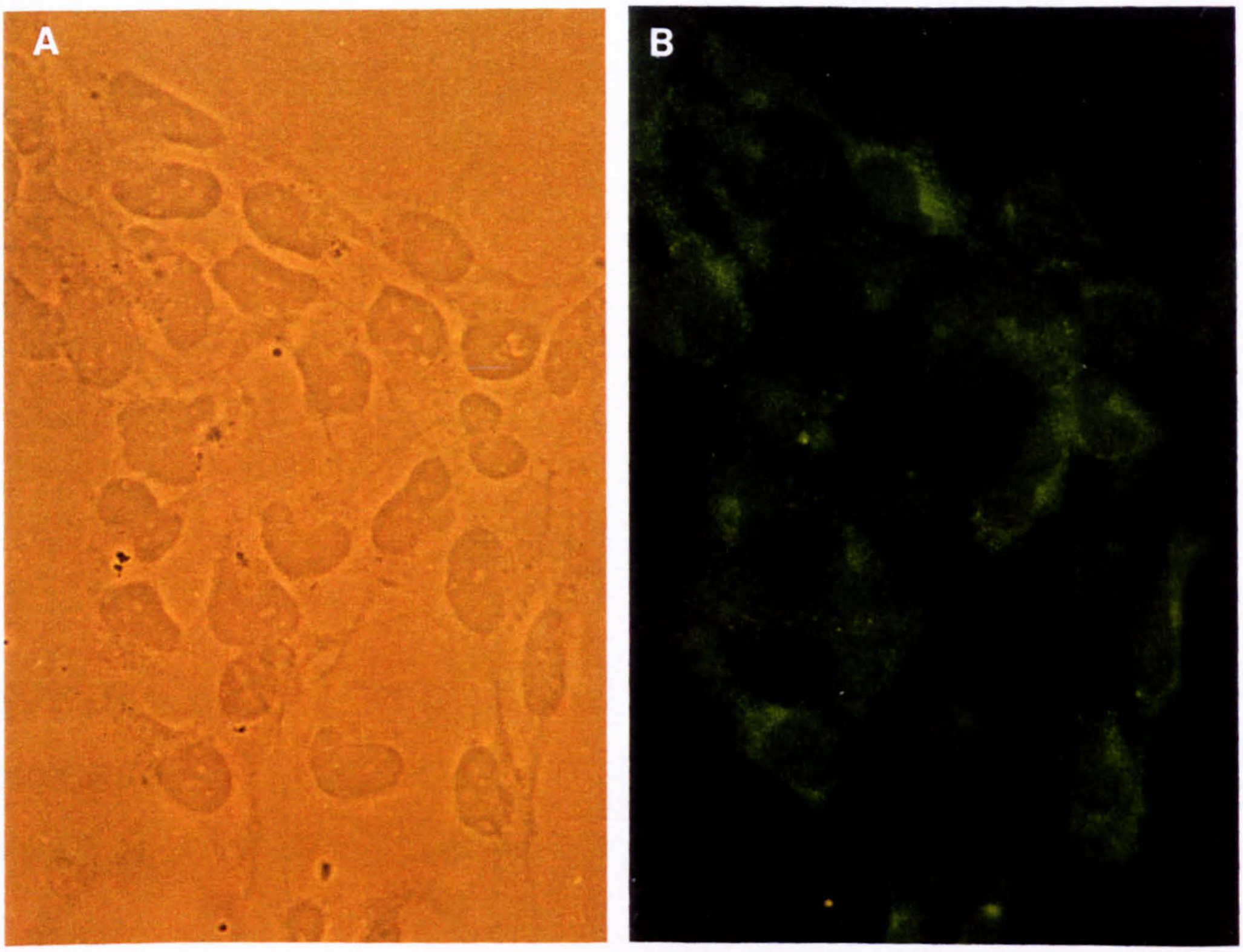

A
120
100
80
60
40
20
0
% SURVIVAL
0
0.1
1
10
1915 ANTIBODY (μg/ml)
B
120
100
80
60
40
20
0
% SURVIVAL
0
0.05
0.5
5
50
114A ANTIBODY (μg/ml)

Figure 3.18 Schwann cell precursors express both ErbB2 and ErbB4

E14 Schwann cell precursors were cultured in defined medium containing FGF plus IGF and forskolin containing medium for 20 hr. The precursors were then stained with anti ErbB2 (B) and anti ErbB4 (D) antibodies. Schwann cell precursors express both receptors (B.D). A and C are the corresponding phase-contrast view. Magnification 800X.


A
B
C
D

CHAPTER 4

INTERACTION OF SCHWANN CELL PRECURSORS AND NEURONS: NEURONAL CONTROL OF SURVIVAL, PROLIFERATION AND MATURATION

Introduction

In the peripheral nervous system (PNS) Schwann cell precursors and Schwann cells are in close contact with axons and appear to rely on them for survival, proliferation and differentiation. The evidence for this close relationship comes from both in vivo and vitro studies.

In vivo, a significant decrease of proliferation in Schwann cells was observed when neonatal rat sciatic nerves were transected which suggested that Schwann cell proliferation in vivo relies at least in part on neuronal mitogens (Komiyama and Suzuki, 1992). In adult rat, transection of peripheral nerve leads to Wallerian degeneration in which Schwann cells undergo de-differentiation and rapid proliferation (Brown and Asbury, 1981; Pelligrino et al., 1986; Clemence et al., 1989; De Vries and Baichwal 1991). When axons regrow into the distal stump, Po and other myelin proteins are induced and the myelin sheath is reformed, indicating that the signal for Schwann cell myelination comes from axons (Aguayo et al., 1977; Bray et al., 1981; LeBlanc et al., 1987; Mitchell et al., 1990). Together, the evidence suggests that in vivo Schwann cells depend on signals from axons for proliferation and differentiation.

In vitro, co-culture of Schwann cells and neurons has been widely used to study the molecular interaction between these two cell types. One of the most active research areas in this field focused on identifying Schwann cell mitogens derived from neurons. Wood and Bunge, (1976) first showed that Schwann cells which were in contact with neurons and their processes rapidly proliferated. Furthermore, a neurite membrane fraction and an axolemma from brain tissue were also reported to stimulate Schwann cell proliferation (Salzer and Bunge, 1980; 1980; Sobue et al., 1984). It was believed that the Schwann cell mitogen was located on the axonal surface (Salzer et al., 1980). It was later found that the neuronal mitogen was a heparin-binding protein which could elevate intracellular cAMP levels in the Schwann cells (Ratner et al., 1984; 1985; 1988), and does not belong to the FGF growth factor family (Ratner et al., 1988). More recently a 50 kDa neuronal membrane associated mitogen was

isolated (Nordlund et al., 1992), but the molecular nature of the axonal mitogens had not been identified until Morrissey (1995) demonstrated that the rat neuronal surface molecule which stimulated both human and rat Schwann cell proliferation could be blocked by anti HRGβ1(heregulinβ-1) antibody, and also showed that the mitogenic response of human Schwann cells to neurons was reduced by anti ErbB2 ($p185^{neu}$) antibody in a co-culture system (Morrissey et al., 1995). This result indicated that HRG\NDF\GGF or HRG\NDF\GGF-like molecules may act as a component of the neuronal mitogen for Schwann cells.

In addition to the active study of neuronal mitogens, efforts have also been made to find the molecular basis of Schwann cell differentiation in the co-culture system. Bunge et al., (1986) reported that in co-culture it is necessary to have ascorbic acid and serum in the medium to induce myelin assembly in Schwann cells. But in terms of Po mRNA and protein induction in co-cultured Schwann cells only serum free defined medium is required (Brunden et al., 1990; Morrison et al., 1991). In addition to Po, PMP-22, another myelin molecule which has recently been shown to be expressed by Schwann cells, was also upregulated by contact with axons in vitro (Spreyer et al., 1991). Oct-6/SCIP (suppressed cAMP-inducible POU protein), a transcription factor probably related to myelin gene regulation was also induced when Schwann cells were co-cultured with neurons (Scherer et al., 1994). Furthermore, it was also found that direct contact between neurons and Schwann cells was not necessary for some upregulation of Po and SCIP genes by neurons (Bolin and Shooter, 1993). Considering all the evidence presented here it is clear that neuronal signals drive Schwann cell differentiation, but that the molecular identification of these signals is still generally unknown.

To date, there have been few studies on the interaction of Schwann cell precursors and neurons. The only evidence comes from studying neuron conditioned medium (NCM). It was found that in not very pure NCM, Schwann cell precursors survived and developed into Schwann cells (Jessen et al., 1994). The molecular basis for this survival event was not known. In the present study highly purified (95%) neuron cultures generated using an immunopanning method are used. The experiments show

that NCM generated from these pure neuronal cultures can significantly rescue Schwann cell precursors from apoptotic death. Furthermore, direct contact between precursors and axons also supports precursor survival. Interestingly, the survival activity in the conditioned medium and generated by direct contact can be markedly diminished by applying soluble ErbB4 protein to the medium or the co-culture. The results presented here, together with previous observations of NDF survival activity, strongly support the idea that NDF is a component of the neuronally derived signal which supports precursor survival. This study also shows that neurons express NDF but that Schwann cell precursors contain much lower levels or no NDF. In addition to survival, axons also stimulate Schwann cell precursor proliferation and promote the maturation and differentiation of these cells in co-culture.

Results

Conditioned medium from purified neurons supports Schwann cell precursor survival

Neuron conditioned medium (NCM) from E19 DRG neurons has been previously reported to support Schwann cell precursor survival. In that experiment the neurons were exposed to serum and mitotic poisons (cytosine arabinoside) several times to kill dividing non-neuronal cells and cultured neurons were kept for more than 10 days before the medium was collected. Meanwhile the purity of the neurons in the culture was not recorded (Jessen et al., 1994).. In order to get highly purified neurons, I devised an immunopanning technique (for detail see Methods). The purity of the neurons generated by this method is 95% ± 5.3 based on counting every single neuron and nonneuronal cell on the coverslips. The pure neurons were cultured in serum free defined medium and only 1 day of culture was necessary to allow neurite outgrowth (Figure 4.1). Therefore, the NCM collected from this culture contains neuron-derived factors which may closely mimic the factors secreted by neurons in vivo. The results showed that Schwann cell precursors cultured in this NCM for 20 hr survived and also showed flattened and group-forming morphology (Figure 4.2). The NCM acted as a dose-dependent survival factor, and pure NCM supported more than 89% Schwann cell precursor survival. Dilution of the pure NCM resulted in a decreasing survival rate of precursors (Figure 4.3). The percentage survival rate in this experiment is similar to that of the previous report using less pure NCM (Jessen et al., 1994). This implies that neurons do secrete soluble factors into the culture medium that supports Schwann cell precursor survival.

Direct contact with neurites also promotes Schwann cell precursor survival

The experiment above showed that soluble factors in NCM supported 89% Schwann cell precursor survival. The NCM was collected from very dense neuronal cultures (100,000-150,000 neurons/300ul medium). In order to test whether neuronal surface

molecules also support precursor survival a co-culture experiment using neonatal DRG neurons and Schwann cell precursors was established with very sparse neuronal cultures (250 neurons/400µl medium). These cultures only contained few (5%) non-neuronal cells. It is believed that in these very sparse neuron cultures it is possible to exclude the effect of soluble survival factors on precursors since the NCM collected from these very sparse neuron cultures was unable to support Schwann cell precursor survival (less than 10% survival). Moreover, the fluorescent dye, 5(and-6) carboxyfluorescein diacetate succinimidyl ester (CFSE), was used to label all the cells in the culture before addition of the Schwann cell precursors. Therefore, the Schwann cell precursors added on the second day to the cultured neurons could be easily distinguished from the few Schwann cells originally present in the neuronal culture because after immunolabelling with L1 antibodies the added precursors are L1 positive and CFSE negative when viewed under the fluorescence microscope. The results generated from this co-culture system therefore, accurately represent the total survival of Schwann cell precursors on neurons. The caculation of survival rate of precursors in these co-cultures was based on the comparison of total mumber of precursors in or not in contact with neurons 20 hr after addition of precursors with that at 3 hr after addition of cells (for detail see Methods). It was found that all precursors not in contact with neuronal cell bodies or neurites had died by 20 hr, while almost all precursors in contact with neurons or neurite at 3 hr survived after 20 hr (Figure 4.4). Schwann cell precursors aligned along the thin neurites had an elongated morphology. Strikingly, contact with a single neurite seemed enough to support Schwann cell precursor survival; a single neuron usually supported the survival of several Schwann cell precursors (Figure 4.5).

NDF is expressed by neurons but not by Schwann cell precursors

Both neuronal surface molecules and soluble molecules present in NCM showed the potential to support survival of Schwann cell precursors. On the other hand, as previously shown, NDFβ can rescue all precursors from apoptosis in vitro (Chapter 3). Therefore, I asked whether NDF was expressed in either neurons or Schwann cell precursors to determine if this molecule might play an role in the interaction of these

cells. The experiments described below used both immunocytochemistry and Western blotting to localize the expression of NDF.

Schwann cell precursors from E14 rat were cultured in FGF-2 (180pM) plus IGF-1 (13nM) medium for 20 hr. Meanwhile neurons dissociated from E14, E15 and new born DRG were cultured in NGF (50ng/ml) containing defined medium for 20 hr. After 2% paraformaldehyde fixation, both neurons and precursors were stained by both polyclonal anti NDF antibody 1915$^{\#}$ and monoclonal anti NDF antibody 5D6A. It was found that almost all neurons at all three developmental stages were labelled, whereas Schwann cell precursors were completely negative (Figure 4.6) (also see Figure 4.1). For Schwann cell precursors, double immuno-labelling with L1 and 1915$^{\#}$ was also used. To further confirm that the staining was specific by using these antibodies, both polyclonal and monoclonal antibodies were pre-absorbed with NDF before application to the cultures. The results showed that the neuronal NDF positive staining was markedly diminished, suggesting that NDF may only be expressed by neurons and not by Schwann cell precursors. In order to identify the isoforms of NDF expressed by neurons, two monoclonal antibodies (114A for NDFβ, 1H7A4 for NDFα) which specifically recognize NDFβ and NDFα c-loops (personnal communication with Dr D. Wen and Dr D. Chang Amgen Inc.), were applied as above. The staining pattern is similar to that of 1915$^{\#}$ and 5D6A, i.e. positive on neurons and completely negative on Schwann cell precursors (Figure 4.7), indicating that neurons express both NDFα and NDFβ isoforms.

In order to confirm the immunocytochemical observations, immunoblotting was used on recombinant NDFβ and NDFα proteins, and on proteins extracted from cultured pure neurons (from newborn DRG), Schwann cells (from newborn rat), Schwann cell precursors (from E14 rat) and brain (from newborn rat). The monoclonal antibody 5D6A which recognized all isoforms of NDF was used to label the proteins. It was found that the both recombinant NDFβ and NDFα showed a marked band at molecular weight 25kDa which matched the molecular weight of these recombinant proteins (NDFβ-2 MW 24,701 , NDFα-2 MW 24,566). The cultured DRG neurons expressed two marked bands at molecular weights around 45kDa and 66kDa, three

relatively weak bands were also observed between 97kDa to 116kDa. Schwann cell precursors did not express detectable NDF bands. Interestingly, in Schwann cells a weak NDF band appeared at molecular weight around 45 kDa, a slightly higher molecular weight than one of the bands seen in neurons (Figure 4.8). These molecular weights match the previous reports in COS-7 cells in which pro-NDF (both α and β isoforms) had a molecular weight at 60-75kDa and 95kDa, whereas, a secreted form of NDF had a molecular weight of 40-45kDa (Wen et al., 1994). Therefore, the data here indicated that neurons produced abundant pro-NDF isoforms and also secreted this protein. Schwann cell precursors did not produce any detectable NDF. Schwann cells may have low levels of secreted NDF forms.

A Soluble ErbB4 extracellular domain blocks both NCM and neuron survival activity

The experiment above showed that Schwann cell precursors survived both in NCM and on neuronal surfaces, and that only neurons but not Schwann cell precursors expressed NDF. It suggests that neurons contain Schwann cell precursor survival molecules which can either be expressed on the neuronal surface or be secreted into the medium. So far, NDF and FGF in the presence of IGF are the only known survival molecules for Schwann cell precursors (Chapter 3; Jessen et al., 1994; Gavrilovic et al., 1995). Meanwhile, these molecules have also been detected at high levels in spinal cord and DRG neurons in early development (Grothe et al., 1991; Bean et al., 1992; Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994). Furthermore, NDF but not FGF, has been shown like NCM to support the long-term survival of precursors (Chapter 3). Together, this suggests that NDF or FGF might act as a neuronal signal to regulate Schwann cell precursor survival. In order to identify the neuronal molecules involved in survival; a soluble ErbB4 protein, so far shown only to bind NDF molecules , was applied to see whether it could block the survival activity of NCM, and in the neuron-Schwann cell precursor co-culture system. Meanwhile an anti FGF-2 monoclonal antibody DG-2 known to block FGF-2 activity in 3T3 cells (Reilly et al., 1989) was also used to see whether it would block the survival activity in the NCM.

In the NCM blocking assay, 2000 Schwann cell precursors were plated on coverslips in low insulin defined medium plus IGF (13nM). Meanwhile, pure NCM was mixed with soluble ErbB4 (1μg/ml and 3μg/ml) proteins. NDFβ2 (40pM) or FGF-2 (180pM) plus forskolin (5μM) were also incubated with soluble ErbB4 protein (1μg/ml to 3μg/ml) as both positive and negative controls. The results showed that in the 20 hr survival assay soluble ErbB4 protein at 3μg/ml blocked 75% of the NCM survival activity (only 25% precursor survival), and lower doses of ErbB4 proteins had smaller effects (Figure 4.9A). The same dose of ErbB4 (3μg/ml) blocked 86% of the NDFβ-2 survival effect (Figure 4.9B), and had no effect on FGF-2 medium (Figure 4.9C). Therefore, it is clear that this soluble protein blocks the survival activity of NDFβ-2, but has no effect on FGF-2 survival activity. The ErbB4 receptor has not been found to bind to any factors other than those of the NDF family (Culouscou et al., 1993; Plowman et al., 1993; Carraway and Cantley, 1994; Tzahar et al., 1994). Therefore, the blocking effect of this protein in NCM is likely to be specific to NDF or NDF-like molecules. Meanwhile the present experiment also showed that ErbB4 protein itself has no toxic effect on Schwann cell precursors since all precursors survive in the medium containing a mixture of FGF-2 and ErbB4 proteins.

To test whether this protein also blocks the survival effect of neuronal surface molecules, the ErbB4 proteins were added three times in 24 hr to a neuron-precursor co-culture (for details see Methods). In these experiments, a new batch of ErbB4 protein was used (Tested in the NDFβ–2 survival assay it was found that the 9μg/ml of the new batch of ErbB4 protein was equivalent to 3μg/ml of the old batch of ErbB4 protein previously applied to the NCM; this may be due to the purity of this protein in different batches). It was found that ErbB4 at 9μg/ml blocked 60% of the neuronal survival effect (Figure 4.10), and this concentration of ErbB4 protein showed no toxic effect on either Schwann cell precursors in FGF-2 medium (about 90% survival) or neurons in NGF medium (97% survival). Therefore, the major survival molecules on the neuronal surface are likely to be NDF-like molecules.

It was noticed that the soluble ErbB4 protein did not block 100% of the survival activity of either NCM or the neuronal surface, indicating that some other molecules in neurons may also be involved in Schwann cell precursor survival. FGF, another molecule expressed on neurons (Grothe et al., 1991; Bean et al., 1992) was also previously reported to support Schwann cell precursor survival in short term culture (Jessen et al., 1994; Garvrilovic et al., 1995). In order to know whether this molecule also contributed to the survival effect in neurons, the NCM was incubated with DG-2, a monoclonal anti FGF-2 antibody, for at least 2 hours before adding to Schwann cell precursors. Both positive and negative control experiments were carried out in FGF-2 plus forskolin and NDFβ2 medium. It was found that DG-2 antibody (40ug/ml) blocked only 25% of survival activity of NCM (Figure 4.11A), whereas, the same concentration of this antibody blocked a 88% of FGF-2 survival activity (Figure 4.11B), and had no effect at all on NDFβ2 (100% survival). This suggested that FGF molecules in NCM may also make a minor contribution to Schwann cell precursor survival.

In co-culture, neurons stimulate Schwann cell precursors DNA synthesis

Previous experiments showed that NCM was unable to stimulate Schwann cell precursor DNA synthesis (Jessen et al., 1994). In contrast, Schwann cells in direct contact with neurons in a neuron-Schwann cell co-culture system appear proliferate massively (Salzer, 1980). To test whether Schwann cell precursors can also proliferate in response to neuronal surface molecules 4000 Schwann cell precursors were co-cultured with 2000 purified DRG neurons in defined medium containing NGF only, for 24 h, 2 days, 4 days, 6 days, 8 days and 2 weeks respectively. BrdU was added to the medium during the last 2 h on each experimental day. It was found that the DNA synthesis of Schwann cell precursors on the first day remained at 13%, which is the same figure previously shown in vivo (Stewart et al 1993). At 4 days, (which is equivalent to E14+4=E18, it is assumed that the E14 Schwann cell precursors cultured with neurons for 4 days might mimic the development of these cells in vivo for 4 days), the number of BrdU positive cells increased to 18% of the

total Schwann cells. This matches the increased Schwann cell proliferation seen at this stage in vivo. Interestingly, after 2 weeks in co-culture, Schwann cell proliferation decreased to 6% (Figure 4.12). Therefore, Schwann cell precursors co-cultured with neurons share a similar proliferation pattern to that of Schwann cell precursors or Schwann cells in vivo. The results also suggest that neuronal surface molecules, unlike NCM, can stimulate Schwann cell precursor DNA synthesis in culture (Figure 4.13).

In co-culture, neurons drive Schwann cell precursor maturation and differentiation as judged by expression of S100, 04, and Po mRNA and protein.

In the proliferation assay described above, it was noticed that Schwann cell precursors co-cultured with neurons underwent a significant morphological change at later stages in vitro. It was found that flattened precursors gradually became bi-polar or tripolar cells, a typical Schwann cell morphology, in 4-7 days co-culture (Figure 4.14), suggesting that Schwann cell precursors in contact with neurons may undergo maturation. To investigate the possible axon-induced maturation and differentiation of Schwann cell precursors I tested whether molecules typical of Schwann cells, but not present on Schwann cell precursors appeared in the co-culture system. Molecules tested in this assay included S100, 04, Po mRNA and protein. The co-culture procedure was identical to that used in the proliferation assay. 4000 precursors were co-cultured with 2000 purified neurons for 24 hours, 2 days, 4 days and 1 week respectively and anti S100 antibody, anti 04 antibody and anti Po antibody were applied to stain the Schwann cell precursors/Schwann cells at each point (Figure 4.14, 4.15, 4.16). It was found that on the first day no S100, 04 or Po positive cells were detected. By day 2, 20% of cells in co-culture expressed S100 (Figure 4.17A), only 5% of cells expressed 04 (Figure 4.17B), and surprisingly about 55% of cells expressed low levels of Po protein (Figure 4.17C). On day 4, almost all cells express S100 protein (83%) and 55% of cells were 04 positive, interestingly the percentage of Po positive cells in contact neurons decreased to 47%, but the Po levels in individual cell were upregulated. By the 7th day, most cells were 04 positive (93%) and the number of Po positive cells went down further to 39%, and most Po positive cells

showed a several fold increase of Po protein levels (Figure 4.14). It is unknown why some cells with low levels of Po expression will eventually become Po negative when these cells are in continued in contact with neurons, while other Po positive cells showed a significant increase of Po levels in 7 days co-culture. One possibility is that different neurons may produce either positive or negative signals to drive Schwann cells to develop into either myelin-forming Schwann cells or non-myelin-forming Schwann cells. Moreover, by using these three different antibodies the neuronal cell bodies and neurites were all unlabelled, indicating that the staining is specific.

To test whether the Po mRNA expression parallels Po protein expression in this system, in situ hybridisation using a digoxygenin labelled riboprobe was employed to detect Po mRNA (Figure 4.18). The Schwann cell precursor-neuron co-culture was set up as above. The results showed that during the first 24 hr no Po mRNA was detected, by day 4, 40% of cells in co-culture were Po mRNA positive and the number of positive cells decreased to 35% during the 2nd week (Figure 4.19). The control experiment, treated with digoxygenin-labelled probe in the sense orientation, was completely negative. These observations indicated that the expression pattern of both Po mRNA and protein is similar when these cells were co-cultured with neurons.

Discussion

Understanding the molecular dialogue between neurons and glia which triggers survival, proliferation and differentiation events in these cells, is an important step towards understanding the development of the PNS. Therefore, identifying one of these signals at the molecular level is a significant step. This study has presented strong evidence that NDF , a neuronal molecule, can act as a neuro-glia signal to control Schwann cell precursor survival. Meanwhile, NDF has also been reported to act as a neuronal mitogen to stimulate Schwann cell proliferation (Morrissey et al., 1995). Together, it seems likely that NDF can function as a key neuronal molecule to trigger several biological events in the cells of the Schwann cell lineage.

In addition to identifying NDF as the neuronal survival factor, evidence was also presented that FGF is involved in neuronally mediated Schwann cell precursor survival, both soluble proteins in NCM and neuronal surface molecules supported Schwann cell precursor survival. Furthermore, Schwann cell precursor proliferation relies on direct contact with axons, and the interaction of Schwann cell precursors and neurons in culture leads to maturation and differentiation of Schwann cell precursors.

Both soluble and surface molecules derived from neurons can act as survival factors for Schwann cell precursors

It has always been interesting to know whether direct neuro-glia membrane signalling is necessary for Schwann cell precursor or Schwann cell survival, proliferation and differentiation. At least for Schwann cell proliferation it is known that secreted molecules from neurons were insufficient to activate proliferation of these cells. Only direct contact with axons caused DNA synthesis in Schwann cells (Salzer et al 1980). But for Schwann cell differentiation towards a myelin phenotype both soluble and neuronal surface molecules are active although the molecules involved in these events are still elusive (LeBeau et al 1988; 1990; Lemke and Chao, 1988; Bolin and

Shooter, 1993). The present experiments demonstrated that both pure NCM and direct contact with axons can rescue almost all Schwann cell precursors from cell death. Therefore, it seems likely that Schwann cell precursor survival and Schwann cell differentiation depend on either soluble neuronal proteins or neuronal surface molecules. Proliferation of cells in the Schwann cell lineage may, in contrast, strictly rely on direct contact with axons.

Although both survival factors in NCM and on the neuronal surface support Schwann cell precursor survival the survival activity generated from these two conditions appears to be quantitatively different although strict comparisons are difficult. In the very sparse cultures of neurons, it was found that a single neuron can support survival of several precursors. In contrast, the NCM in this study was generated from 100,000-150,000 neurons which seems just enough to support precursor survival since dilution of the NCM resulted in significant decrease of survival in these cells. It is unclear what causes these different survival potentials, the simplest explanation for this is that neuronal surface may have a higher levels of survival molecules than that NCM. The other alternative possibility is that the survival molecules expressed in NCM or on neuronal surface are different.

Identification NDF as the key neuronal molecule for precursor survival

Although it seems likely that the neuronally derived molecules for Schwann cell precursor survival are both soluble and cell surface molecules the molecular nature of the survival factor was previously unidentified. The studies described in Chapter 3 demonstrated that NDF has the same biological potential as NCM to support Schwann cell precursor short-term and long-term survival, and to promote maturation of these cells (Dong et al., 1995). The present experiments showed that a soluble ErbB4 extracellular domain, a highly specific receptor, which, as far as is known, binds only to NDF\HRG\GGF molecules, significantly blocked both neuron conditioned medium and neuron surface mediated Schwann cell precursor survival. Meanwhile, immunocytochemistry showed that only neurons and not Schwann cell precursors

express NDF. The Western blot analysis of proteins from both neurons and Schwann cell precursors in this study also indicated that only neurons produced pro-NDF and secreted forms of NDF. Together, the evidence here implies that NDF acts as a neuron-glia signal to promote Schwann cell precursor survival, and that the survival molecules both in NCM and on the neuronal surface are likely to be NDF or NDF-like molecules. More recently, it is found that in NDF (also called neuregulin) knockout mouse embryos, Schwann cell precursors in the trunk appear severely reduced in number (Meyer and Birchmeier, 1995). These observations further indicated that NDF or NDF-like molecules may play a important role in the modulation of precursor survival and its fate decisions.

Interestingly, the present data also showed that Schwann cells but not Schwann cell precursors express low amounts of NDF in a Western blot assay. It gives rise to the possibility that the survival of Schwann cells in routine medium may depend on the endogenous NDF which is expressed in these cells, whereas Schwann cell precursors which do not express NDF undergo programmed cell death in culture. It is unclear, however, whether the NDF expressed in Schwann cells is produced by the Schwann cells themselves, since no high molecular weight bands, probably indicative of pro-NDF, were detected in Schwann cells by Western blot. Therefore, the secreted form of NDF which is detected in Schwann cells may still be derived from neurons.

The blocking of NDF survival activity both in NCM and co-culture results in only 60-75% cell death. Some of precursors can still survive, suggesting that other molecules may also be involved in regulation of precursor survival. In the oligdendrocyte lineage, multiple signals are necessary for the long-term survival (Barres and Raff, 1994). This probably reflects the general advantages of combinatorial control in the animal kingdom. Experiments presented here further showed that FGF-2 blocking antibody diminished Schwann cell precursor survival by 25% in NCM suggesting that FGF-like molecules in neurons may also contribute to Schwann cell precursor survival. Therefore it is possible that multiple signals exist in the PNS to support Schwann cell precursor survival although the multiple factors are not necessary to

support precursor survival in vitro. However, NDF is likely to play a major role in the neuro-glia signalling which mediates Schwann cell precursor survival.

The proliferation of Schwann cell precursors depends on direct contact with neurons in vitro

The data present here show that Schwann cell precursors in contact with neurons or neurites in co-culture are stimulated to synthesize DNA, and that over a period of time, the DNA synthesis pattern by precursors in this co-culture system is similar to that of Schwann cell precursors in vivo (Strewart et al., 1993), suggesting that the development of Schwann cell precursors in vivo can be mimicked in vitro in this co-culture system, at least in terms of the proliferation. Although this study gives no direct evidence to identify the neuronal mitogen for Schwann cell precursors, three relevant pieces of evidence suggest that NDF may also act as an axonal mitogen for Schwann cell precursors. Firstly, NDF has been shown to stimulate DNA synthesis in isolated Schwann cell precursors in culture (Chapter 3). Secondly, NDF\HRG\GGF has recently been identified as a component of the axonal mitogen of human and rat Schwann cells (Morrissey et al., 1995). Thirdly, both neurons and axons were shown to express high levels of NDF protein. Considering NDF as a neuronally-derived mitogenic signal it raises the further question as to why NCM does not activate Schwann cell precursor proliferation. It is possible that the concentration of HRG\NDF\GGF in NCM is too low to activate proliferation events, since previous experiments have shown that only high concentrations of NDF act as a mitogen for Schwann cell precursors (Chapter 3).

The lack of mitogenic activity in NCM, because of the low concentration of NDF, may also affect Schwann cell proliferation since blocking direct contact between neurons and Schwann cells by a permeable collagen diaphragm prevented division in these cells (Salzer et al., 1980). It is unclear why direct contact with neurons promotes DNA synthesis in these cells. Two possibilities exist to explain the fact that the neuronal surface membrane has mitogenic activity for both Schwann cell

precursors and Schwann cells whereas NCM does not. First, the neuronal surface may recruit a high local concentration of NDF which stimulates proliferation in these cells. The evidence for this hypothesis is based on the fact that NDF is synthesized as a transmembrane protein, and the pro-NDF isoforms could be expressed in association with the membrane, and could also be cleaved from the membrane (Marchionni et al., 1993; Wen et al., 1994), in which case the soluble protein could also bind to cell surface heparin and other membrane-associated proteoglycans. Therefore, theoretically, the NDF concentration at neuronal membrane could be higher than that in NCM. Moreover, the Western blotting in this study suggested that neurons expressed abundant pro-NDF isoforms and the 45kDa secreted isoform. Furthermore, the survival experiments discussed above also indicated that the concentration of the survival molecule, NDF, at the neuronal surface might be higher than that in NCM. Together, it is possible that the NDF concentration at the neuronal surface is higher than that in NCM, which leads to proliferation of Schwann cells and Schwann cell precursors on neurons. The other possibility is that the mitogenic activity of neuronal surfaces for these cells may depend on a combination of several factors which includes NDF and some other factors which may not be cleaved and secreted into medium.

Schwann cell precursors in contact with axons generate Schwann cells

Schwann cell precursors in vivo eventually develop into Schwann cells. This cell conversion involves a change in several cellular proprieties, including expression of the Schwann cell marker S100, survival ability in routine medium and a morphological change (Jessen and Mirsky, 1991; 1992; Jessen et al., 1994). The experiments described here showed that Schwann cell precursors in contact with neuronal cell bodies or neurites were induced to express S100, 04 and Po. The percentage of S100 positive cells at day 4 in co-culture (82%) is similar to that of S100 positive cells in vivo at E18 (96%) (Jessen et al., 1994). Meanwhile, the 04 expression of these cells in 1-7 days co-culture period is also well matched to that seen in vivo. These results indicate that Schwann cell precursors in contact with neurons generate Schwann cells. The morphological change of precursors in co-

culture also suggests that conversion has occured. Combined with previous observations that NCM can also drive precursors to develop into Schwann cells (Jessen et al., 1994), the data presented here provide substantial support for the idea that neuronal derived signals drive Schwann cell precursor maturation.

It is clear that Schwann cells in co-culture with neurons can be induced to express relatively high levels of myelin protein Po (Brunden et al., 1990; 1992; Morrison et al., 1991). Therefore, it is of interest to know whether Schwann cell precursors, after conversion to Schwann cells, could also be induced to differentiate further. This study also indicated that at day 2, almost 55% of cells in co-culture express low levels of Po protein, and later about 40% of cells were induced to express high levels of Po. It is unclear what is the function of this low levels of Po protein at early stage, at least, it is known that in chicken, Schwann cell precursors also express Po protein (Bhattacharyya et al., 1991; 1994). Neverthless, Schwann cell precursors after conversion to Schwann cells could also be induced to express high levels of Po, indicating that neuronal signals not only drive Schwann cell precursor maturation but also promote its differentiation. Alternatively, Schwann cell precursors may only require neuronal survival signals, and subsequently, Schwann cell precursors may control their maturation and differentiation autonomously. To date, there is no direct evidence in this regard.

Figure 4.1 Purified DRG neurons and their expression of the NDF

Newborn rat DRGs were dissociated and immunopanned in Thy1.1 coated dishes, and 95% pure DRG neurons were obtained and plated at a density of 100,000-150,000 neurons/300µl with 50ng/ml NGF (A). The neuron conditioned medium was collected from these pure neuron cultures on the 2nd and on the 3rd day. In another experiment, the same purified neurons were plated on coverslips and stained with anti NDF antibody 5D6A. Neurons from newborn DRG express NDF (B). Magnification 600X

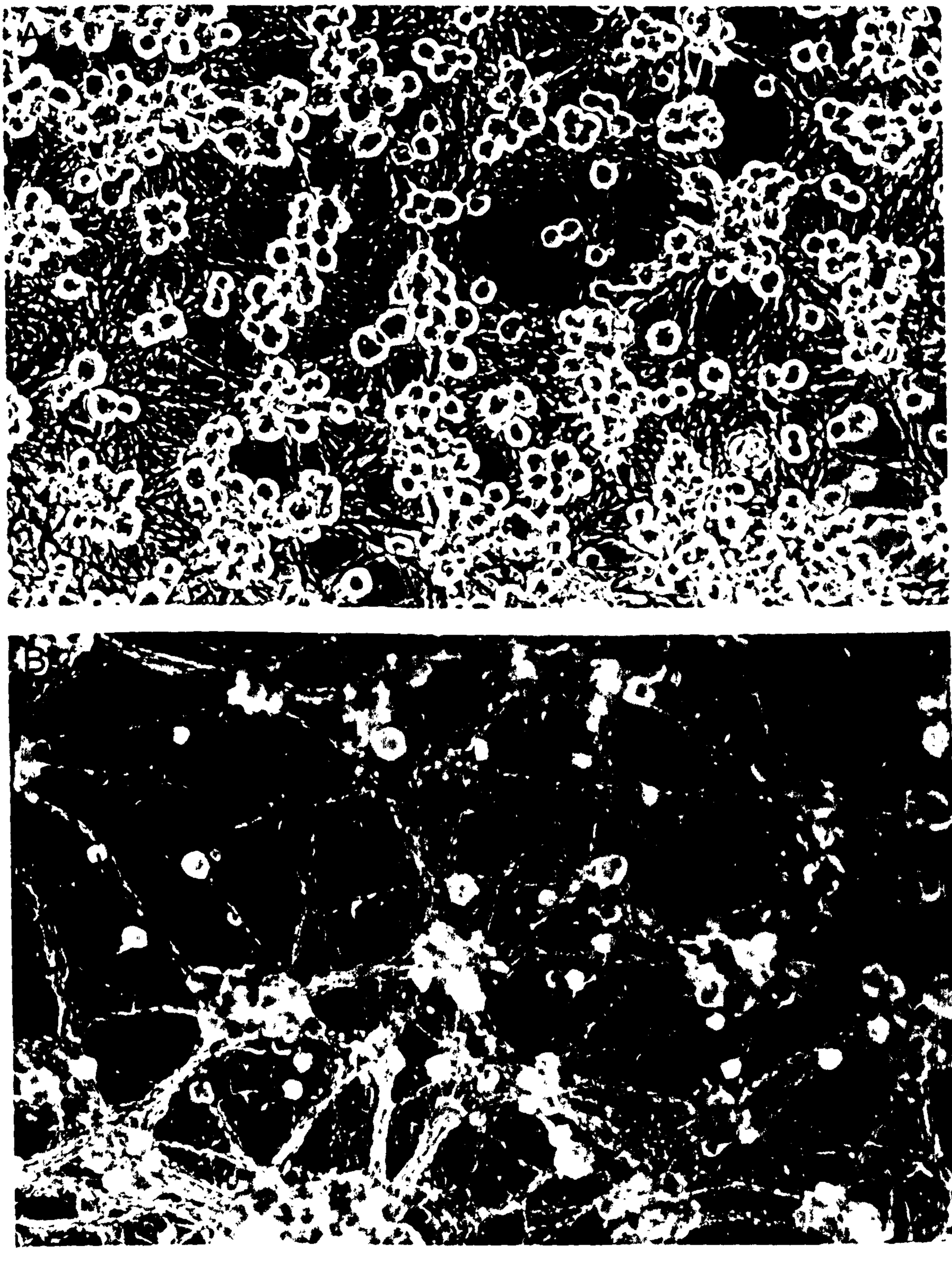
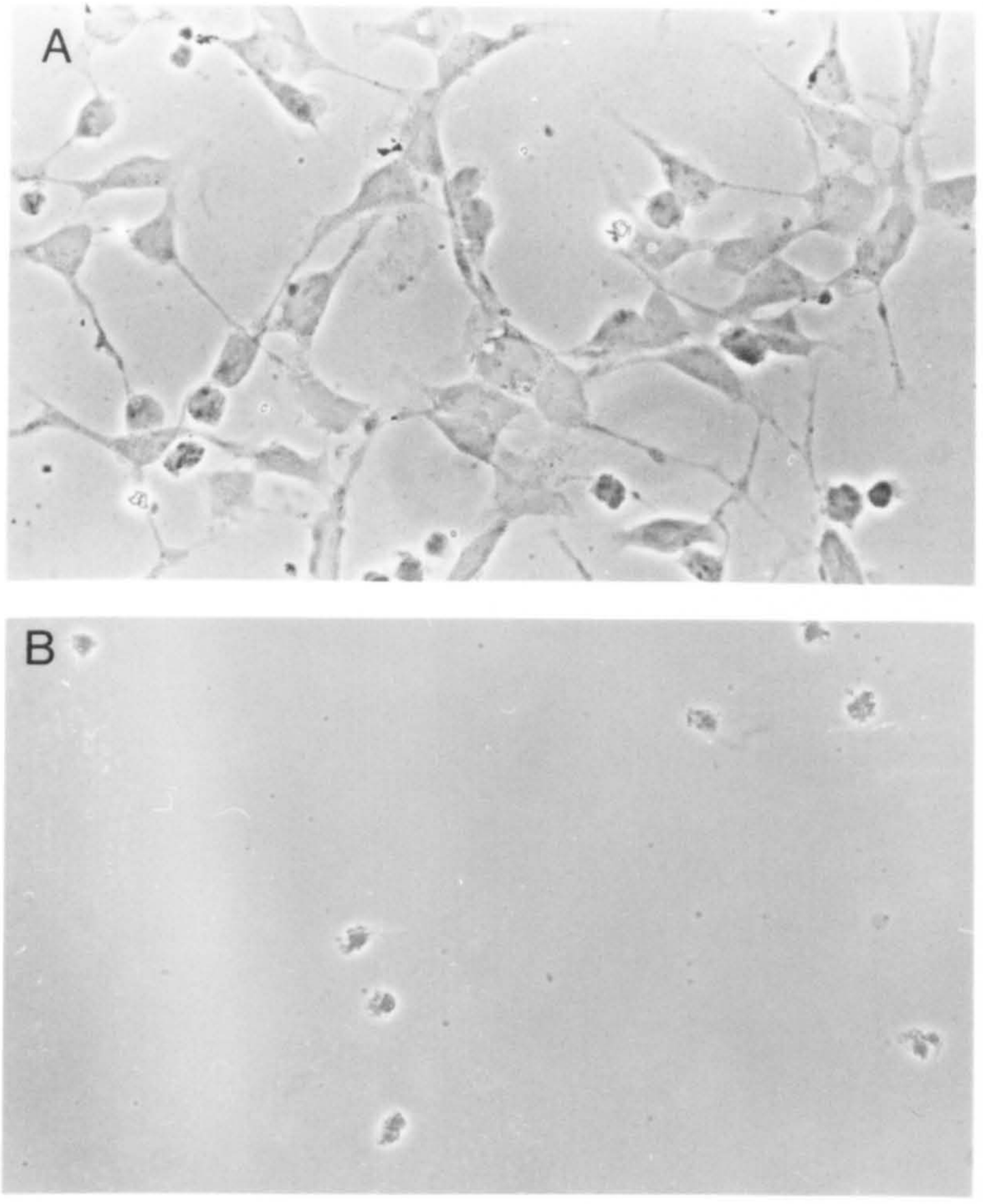
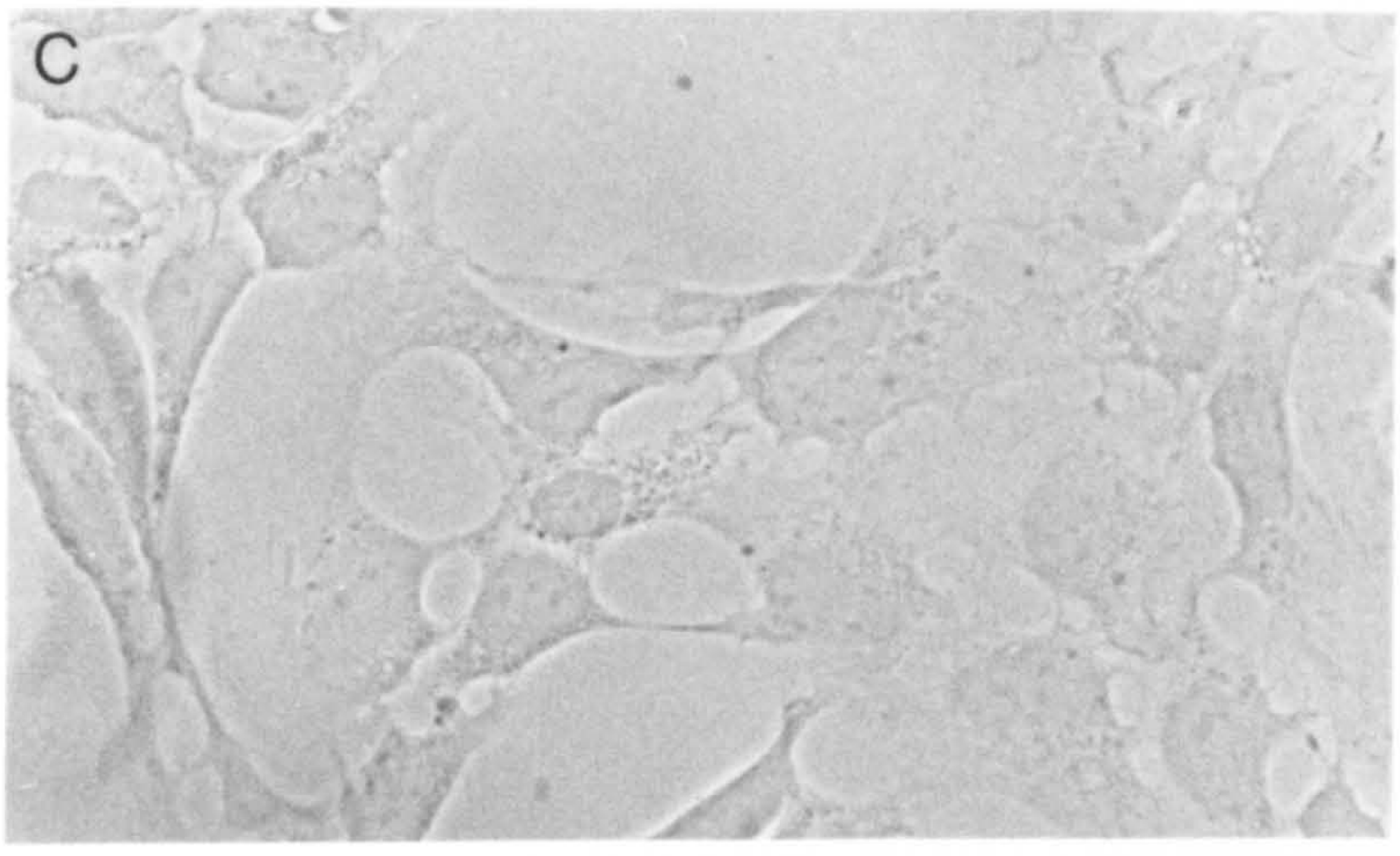

B

Figure 4.2 Pure neuron conditioned medium rescues precursors from apoptotic cell death

Schwann cell precursors from E14 nerves were plated on coverslips in 10 µl defined medium containing IGF-1 (13nM) for 3hr (A), then the precursors were topped up either defined medium with IGF (B) or neuron conditioned medium (C,D) for total 20 hr. L1 antibodies were used to label the surviving precursors (D) after a total 20 hr assay. Magnification 600X


A
B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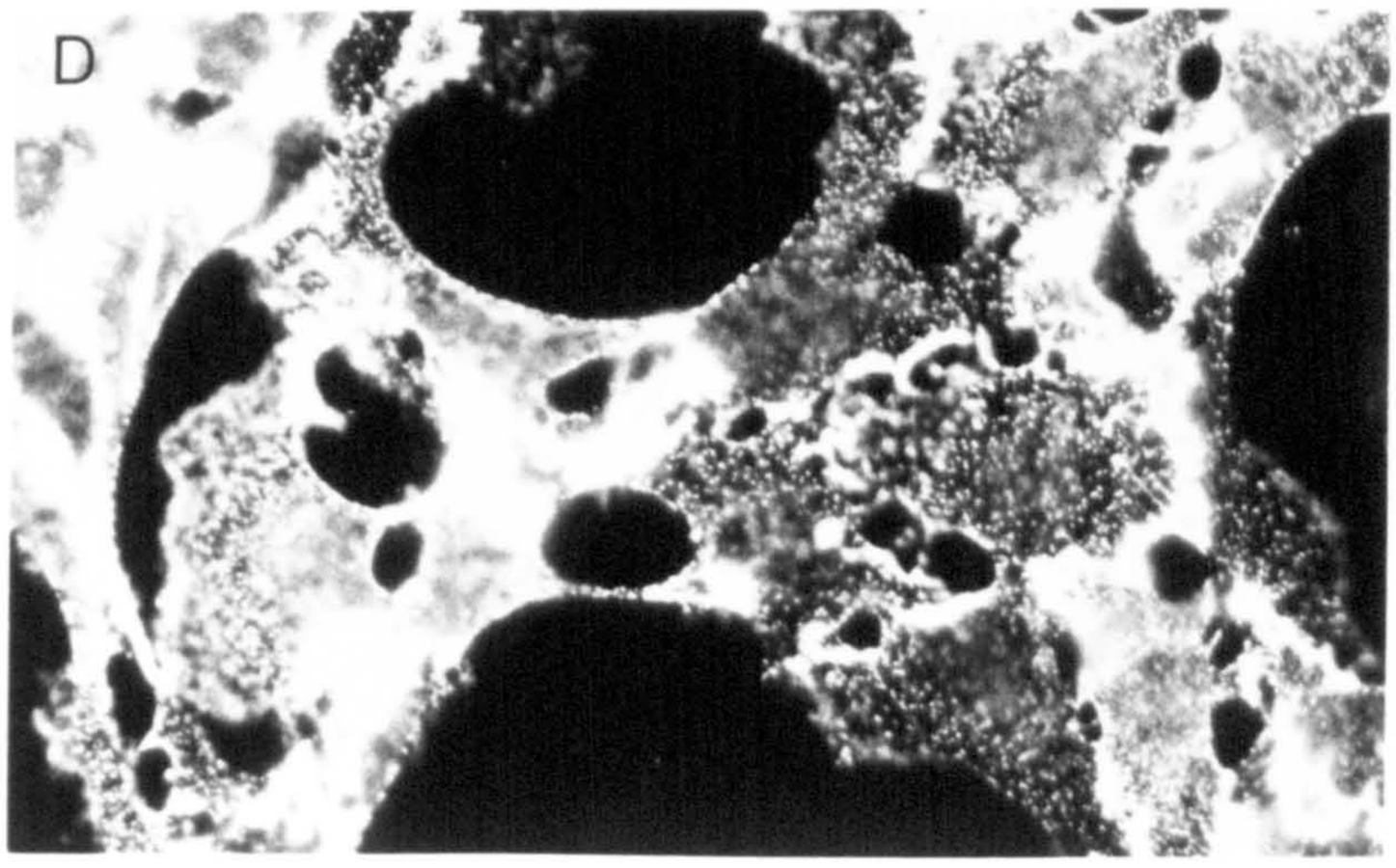

D

Figure 4.3 Neuron conditioned medium supports Schwann cell precursor survival

Various concentrations of neuron conditioned medium were used on Schwann cell precursors for a 20 hr assay. The surviving precursors were labelled with L1 antibody and counted. The results show that neuron conditioned medium acts as a dose-dependent survival factor for precursors.

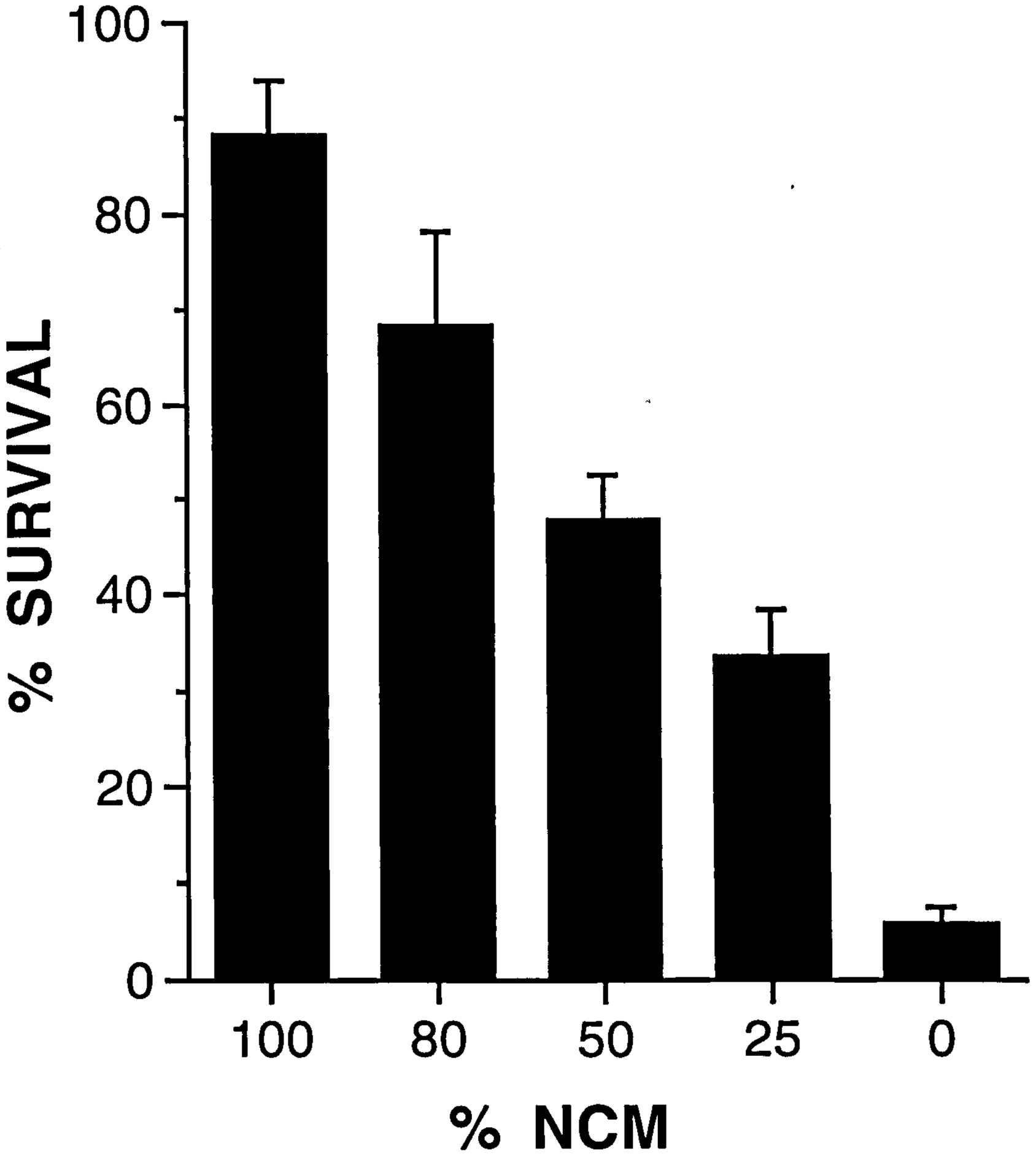

100
80
60
40
20
0
% SURVIVAL
100
80
50
25
0
% NCM

Figure 4.4 Direct contact with the neuronal surface supports Schwann cell precursor survival

Purified DRG neurons were obtained from neonatal animals by an immunopanning method. 250 neurons pre-labeled with CFSE were cultured in 100 μl of defined medium with 50ng/ml NGF for 18-20 hr. 2000 Schwann cell precursors were then plated on cultured neurons on the second day for a further 24 hr of culture. The precursors were labelled with anti L1 antibodies. The total number of precursors in contact with or without neruones or neurites as counted at the 3 hr and 24 hr point after plating. The survival percentage refers to the total surviving precursors at the 24 hr point in comparison with that at the 3 hr point. The results show that precursors in contact with neurons or neurites survive.

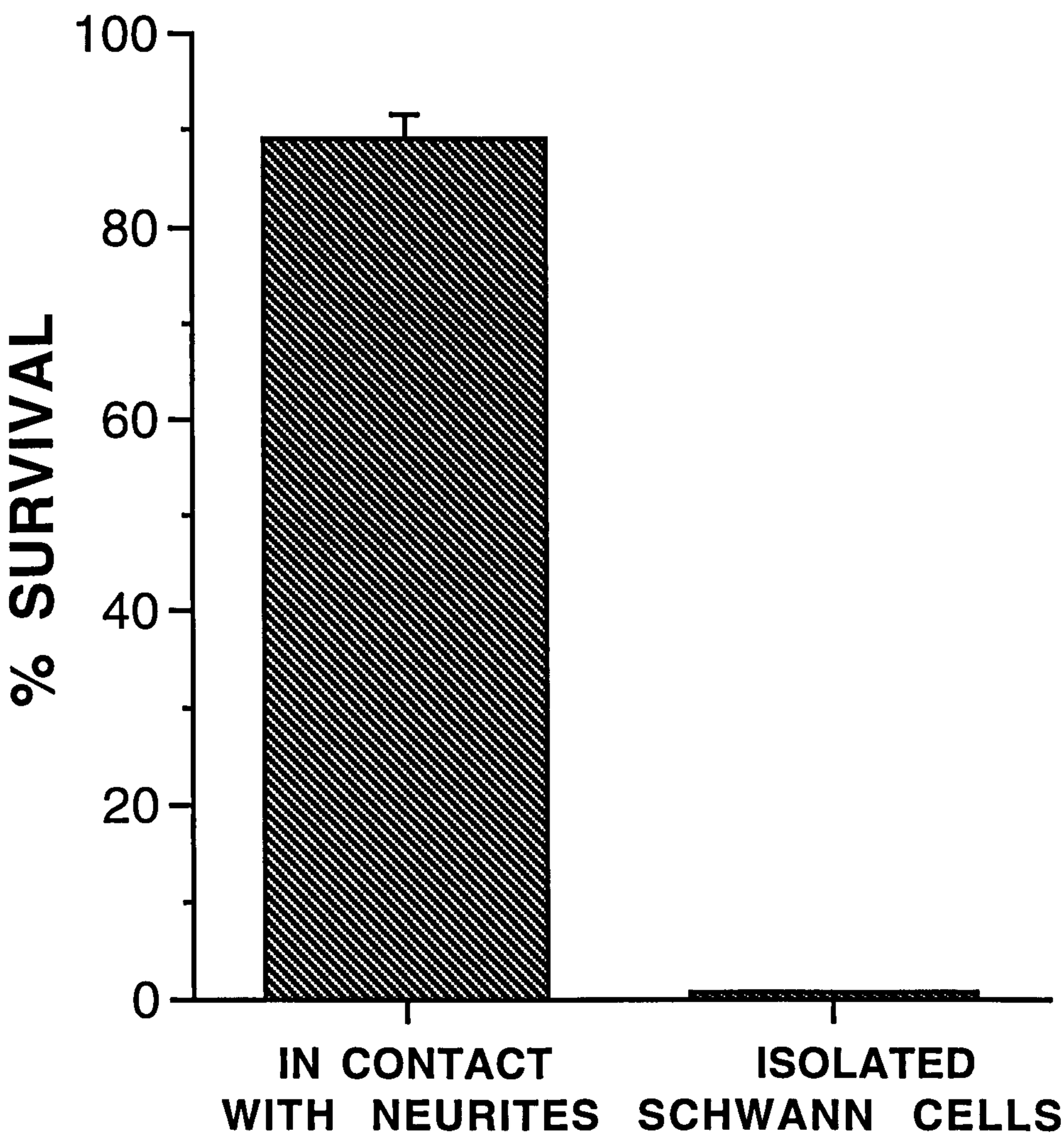

100
80
60
40
20
0
% SURVIVAL
IN CONTACT WITH NEURITES
ISOLATED SCHWANN CELLS

Figure 4.5 Surviving precursors in contact with neurites

E14 Schwann cell precursors were co-cultured with purified neurons in defined medium containing NGF 50ng/ml for 20 hr. The precursors in contact with neurites survive and a single neuron supports survival of several precursors. (A) phase-contrast of a single neuron with several precursors in 20 hr culture. (B) neuron is labelled with CFSE. (C) L1 labels on both precursors and neurons. Magnification 800X

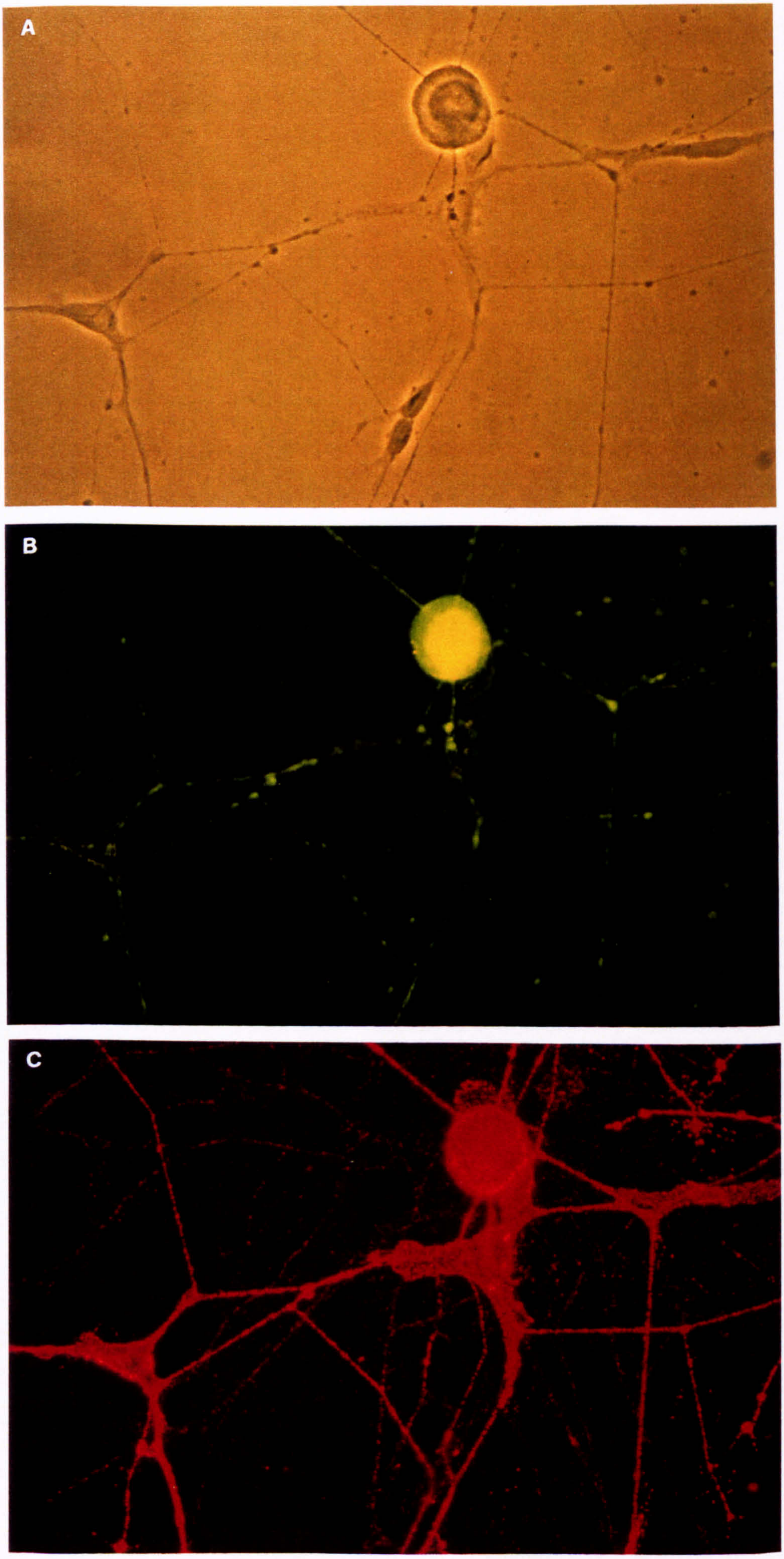
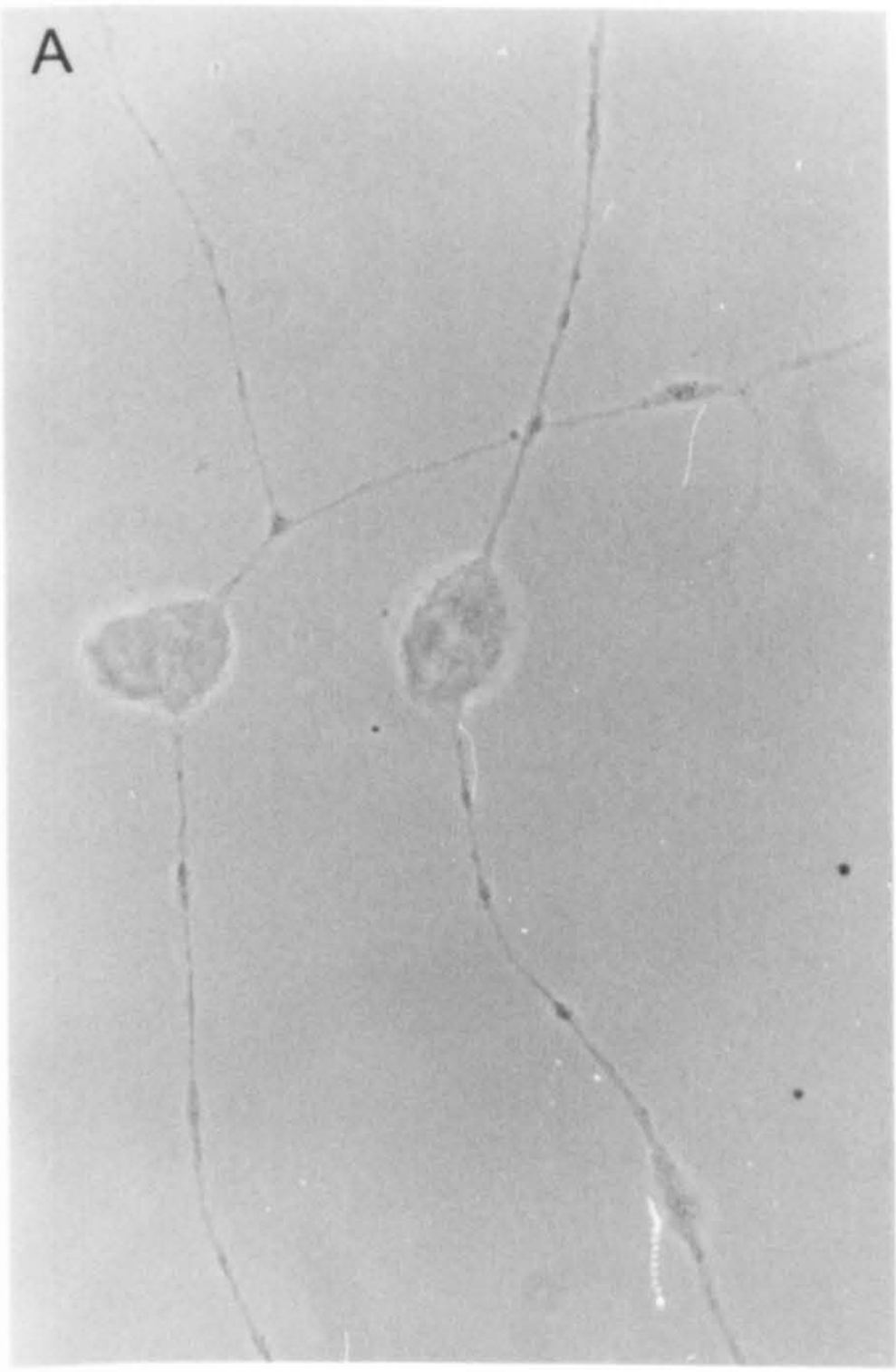
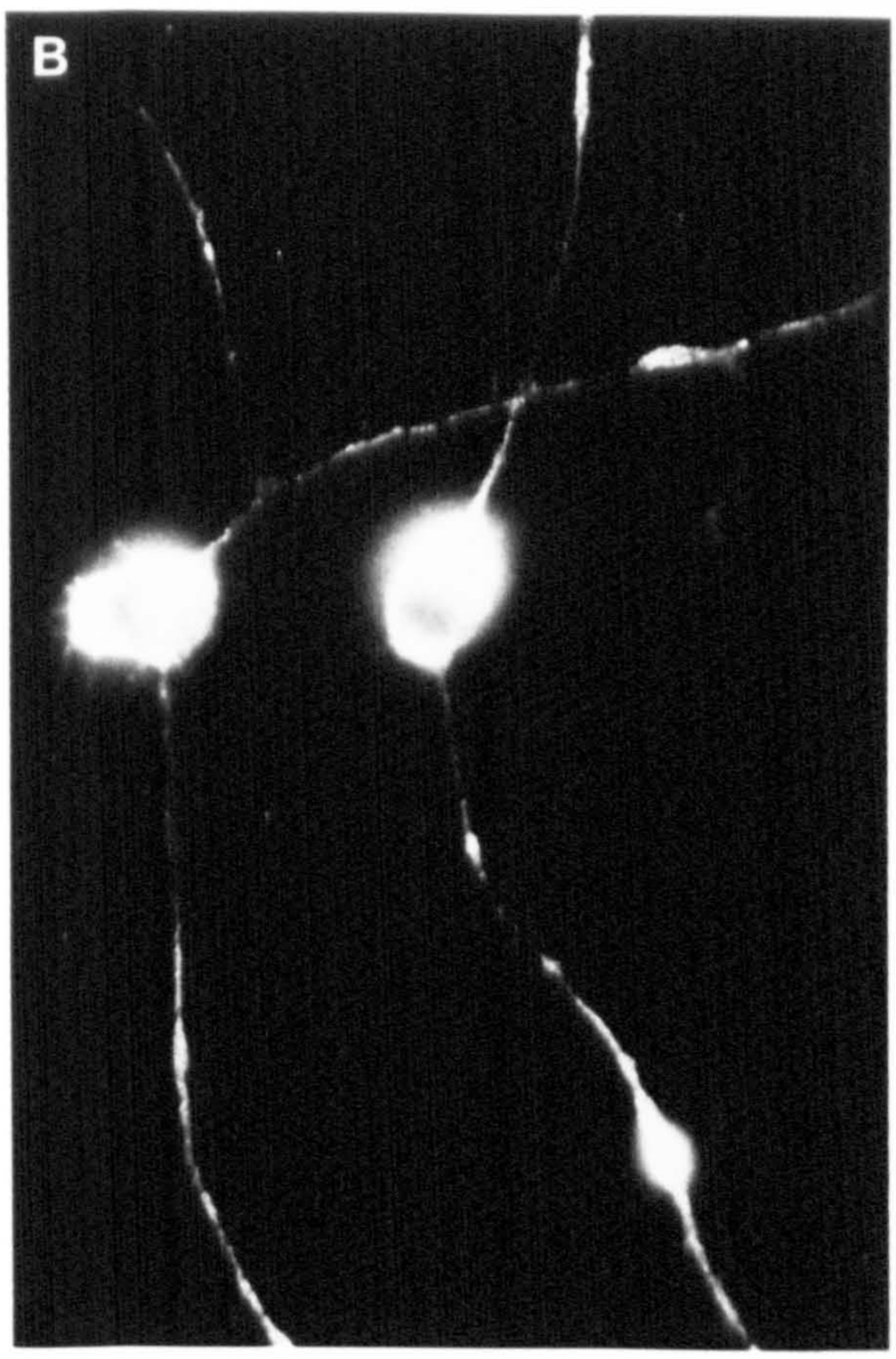

A
B
C

Figure 4.6 Neurons, but not Schwann cell precursors, express NDF

Anti NDF monoclonal antibody 5D6A was used to stain both E14 neurons (A,B) and E14 Schwann cell precursors (C,D). The results show that both neuronal cell bodies and neurites are NDF positive (B), while precursors are NDF negative (D). Magnification 700X


A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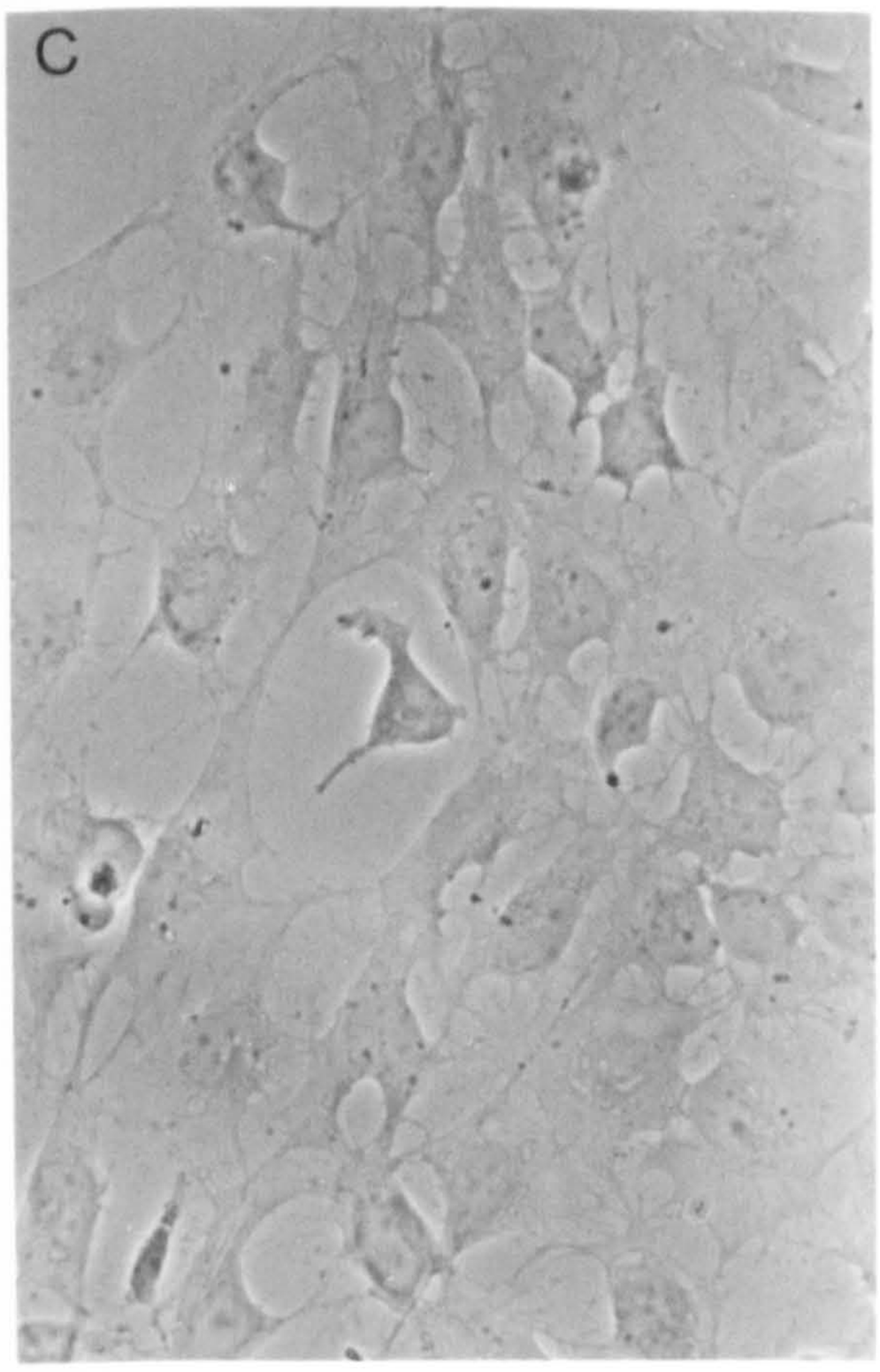

C

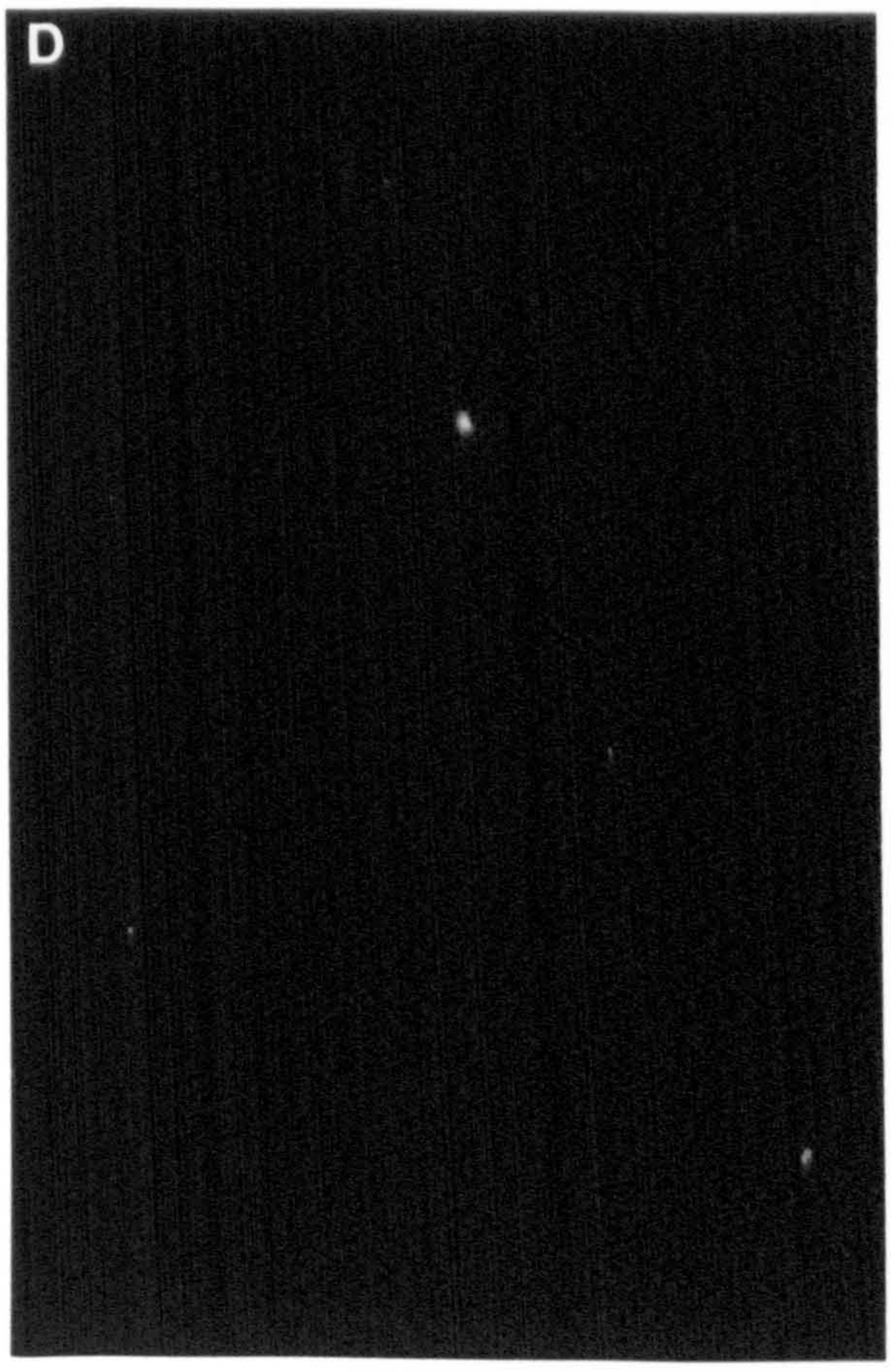
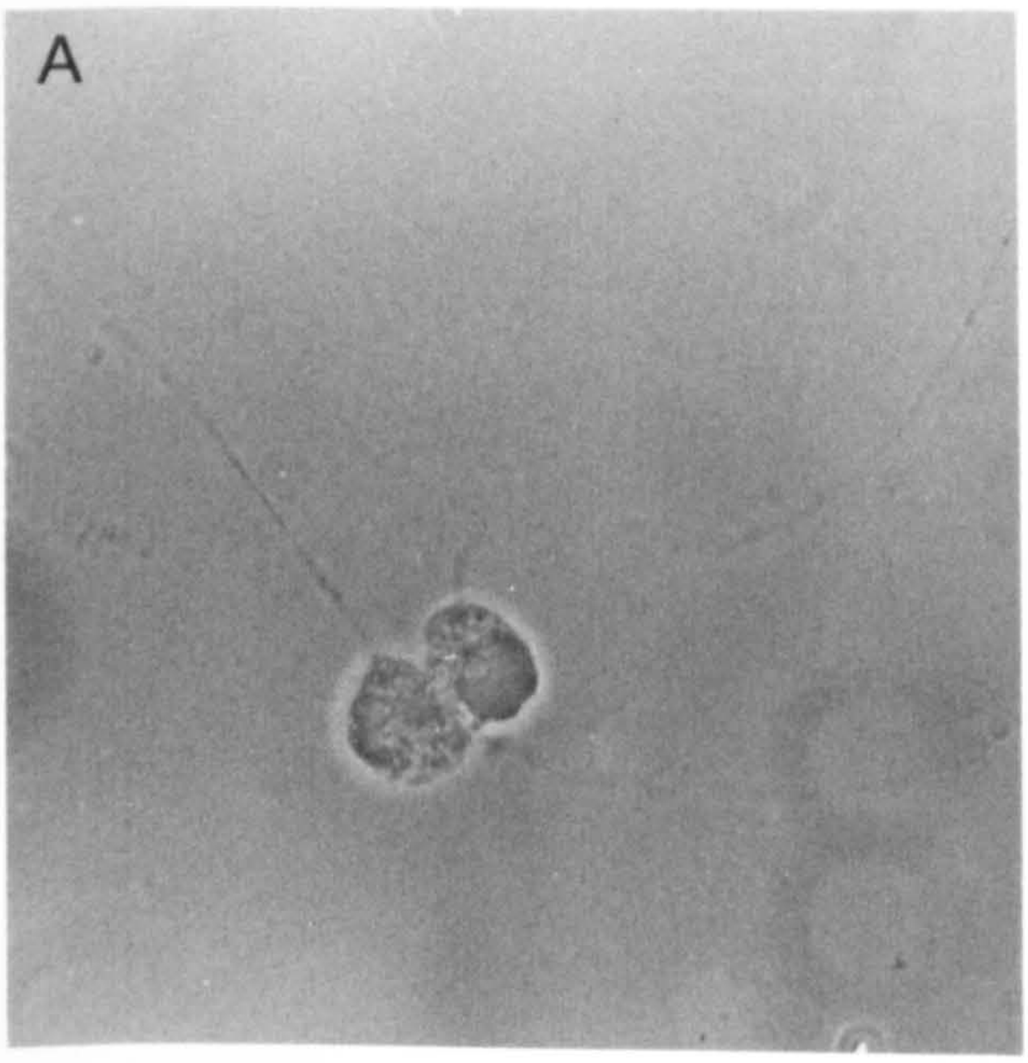
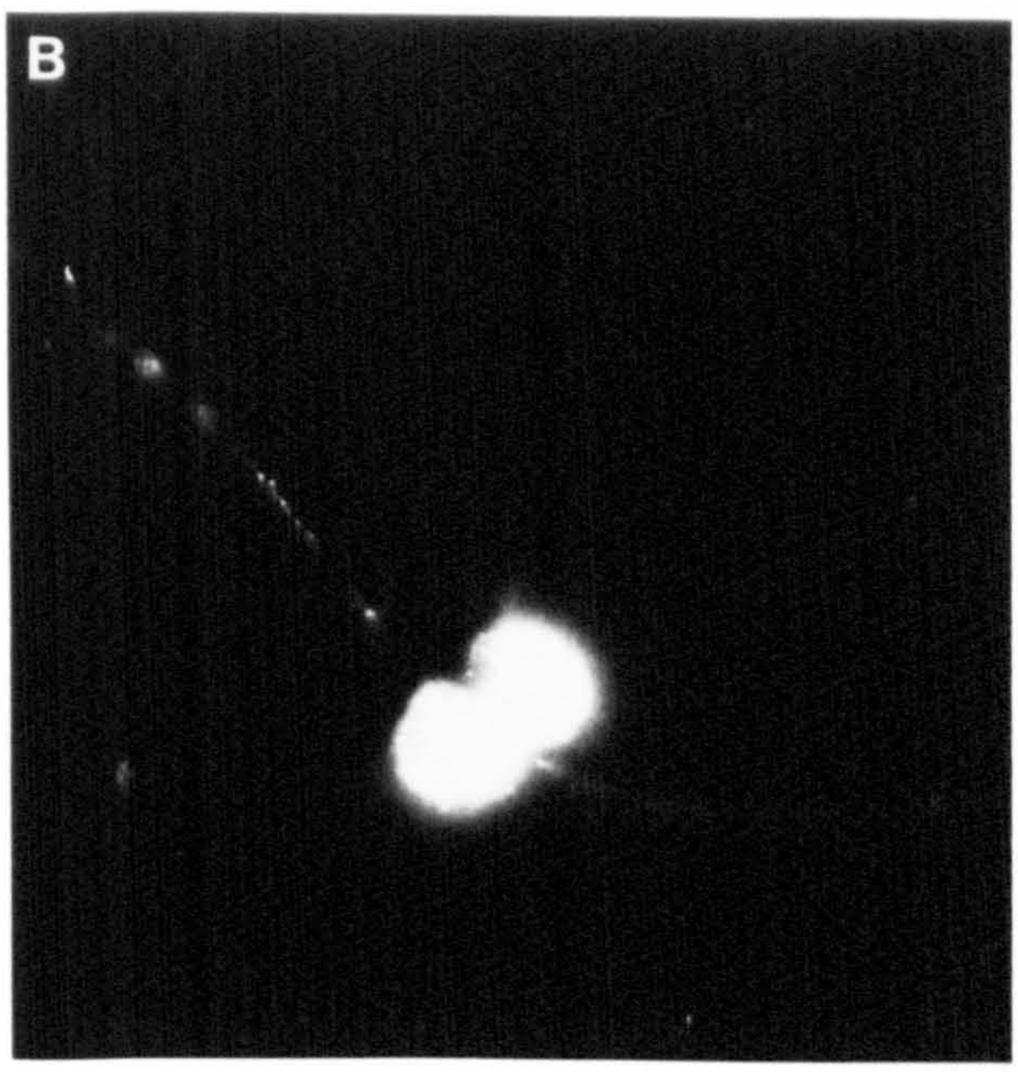
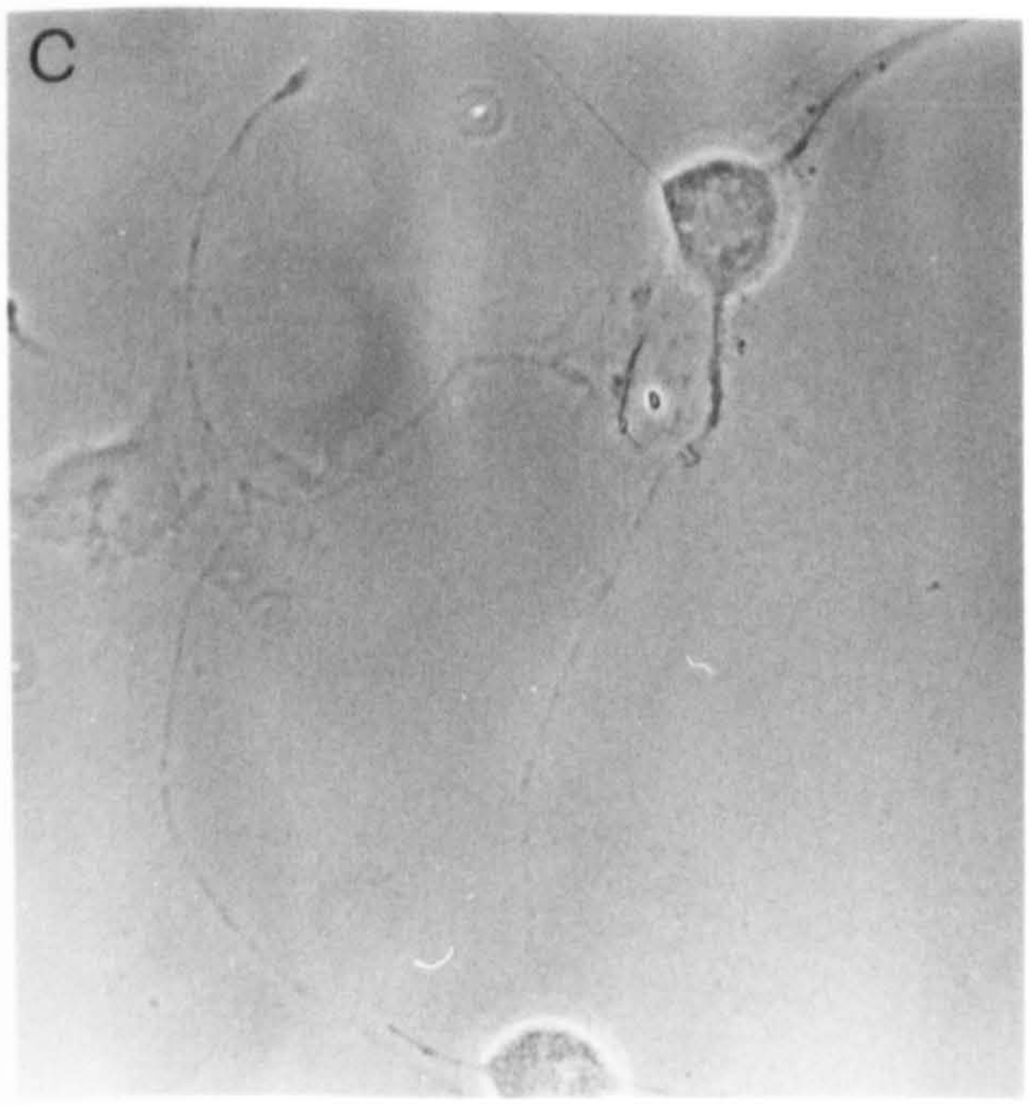

D

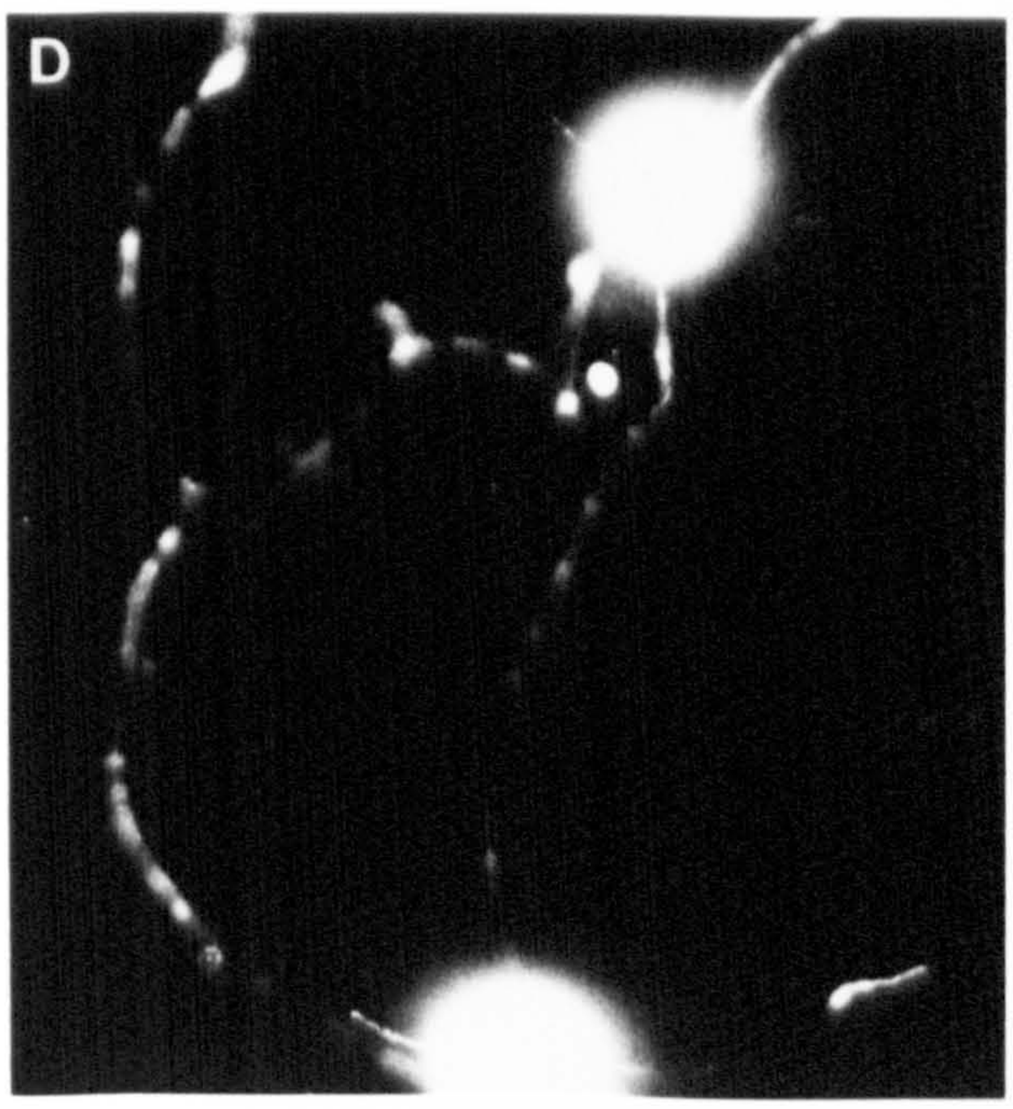
Figure 4.7 Neurons express both NDFβ and NDFα isoforms.

Neurons from E15 rat DRG were stained with anti NDFβ monoclonal antibody 114A and anti NDFα monoclonal antibody 1H7A4. The results show that both neuronal cell bodies and neurites are NDFβ (B) and NDFα (D) positive. A and C are the corresponding phase-contrast views. Magnification 700X


A


B


C


D

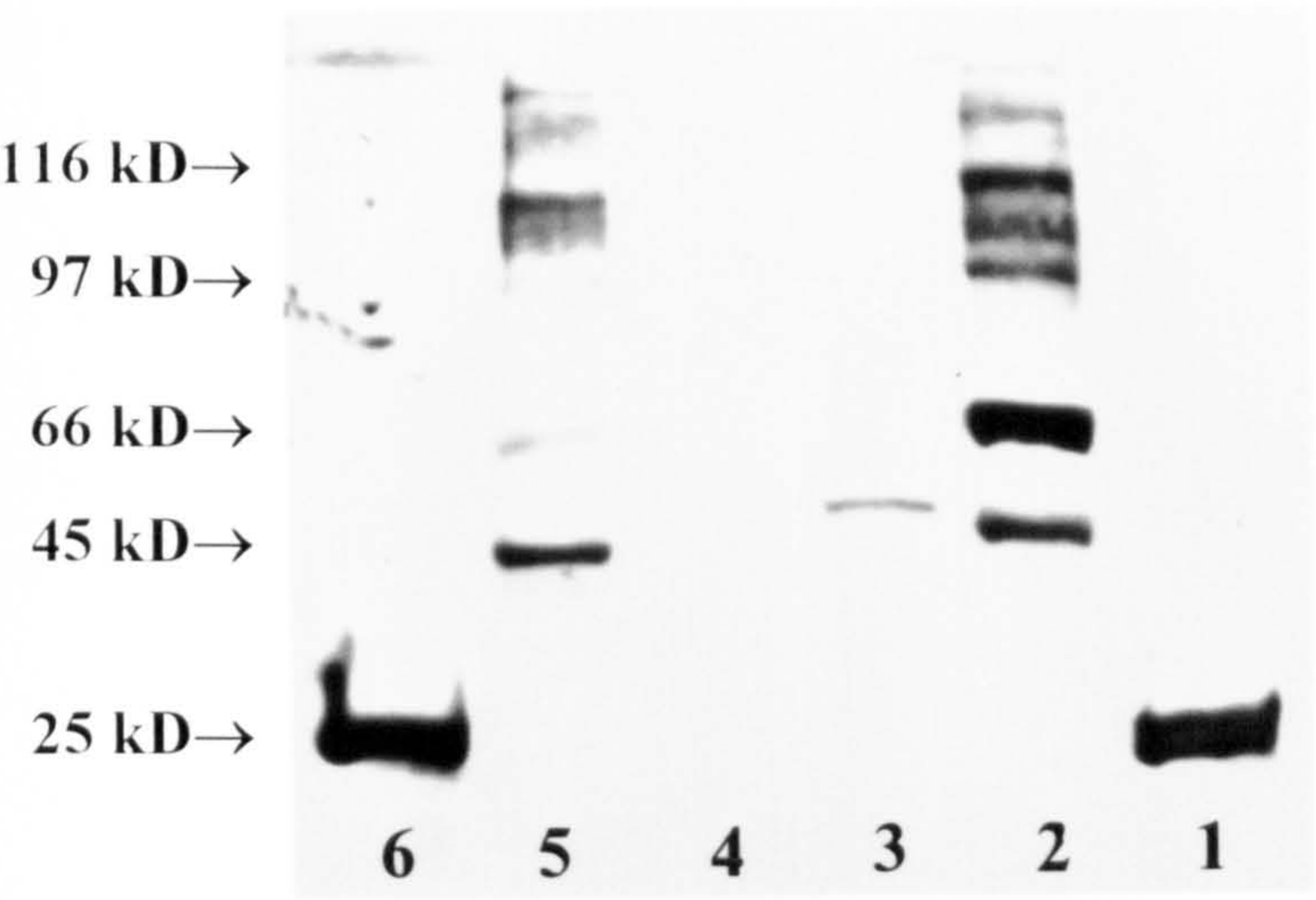


Figure 4.8 NDF expression in different cell types and tissues by Western blot

Lane1: 100ng recombinant NDFβ-2 protein
Lane2: 20μg protein extracted from purified and cultured newborn rat DRG neurons.
Lane3: 20μg protein extracted from cultured newborn rat Schwann cells.
Lane4: 20μg protein extracted from cultured E14 rat Schwann cell precursors.
Lane5: 20μg protein extracted from newborn rat brain tissue.
Lane6. 100ng reconbinant NDFα-2 protein.

Schwann cell precursors and Schwann cells were cultured in defined medium containing FGF-2 (180pM) plus IGF-1 (13nM) for 20 hr and neurons were cultured in defined medium containing NGF (50ng/ml) for 20 hr. After washing in PBS, proteins were extracted from the culture dish. The extracted proteins were then loaded on an 8% acrylamide gel and monoclonal antibody 5D6A was used to detect the NDF protein.

Figure 4.9 Soluble ErbB4 protein blocks NCM and NDF survival activity

Soluble ErbB4 protein blocks the survival activity in pure neuron conditioned medium (A) and NDFβ-2 containing medium (B). It has no effect on FGF-2 containing medium (C). The experiment was based on the 20 hr precursor survival assay as described before.

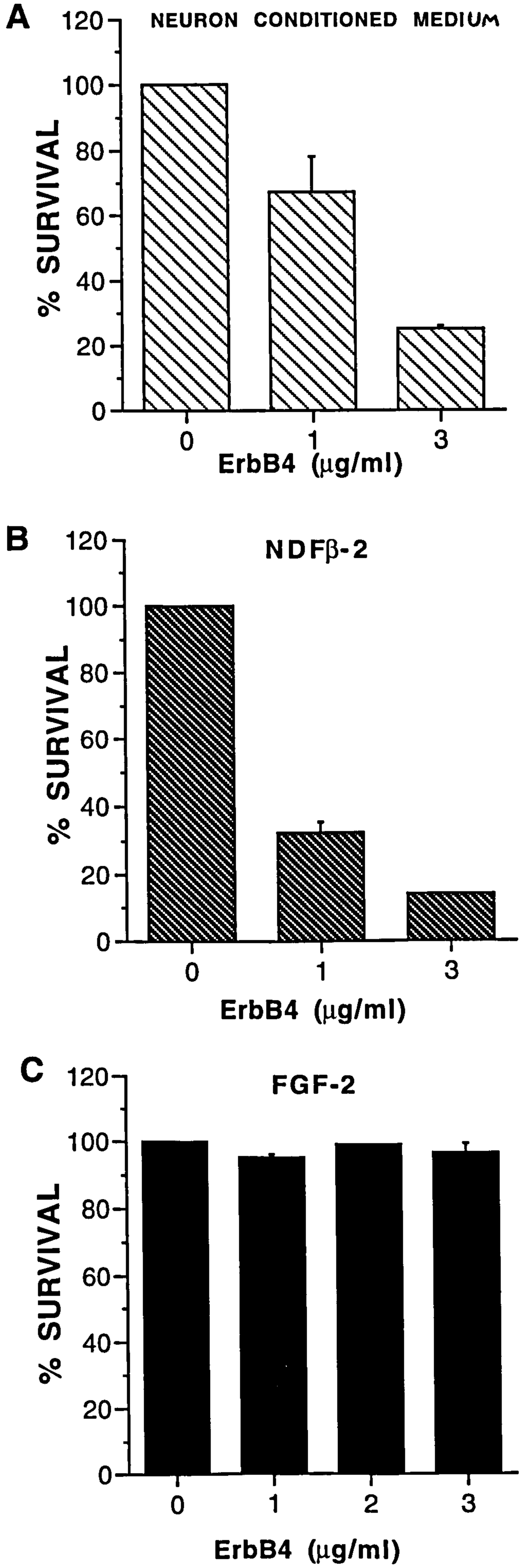

A
NEURON CONDITIONED MEDIUM
% SURVIVAL
120
100
80
60
40
20
0
0
1
3
ErbB4 (μg/ml)
B
NDFβ-2
% SURVIVAL
120
100
80
60
40
20
0
0
1
3
ErbB4 (μg/ml)
C
FGF-2
% SURVIVAL
120
100
80
60
40
20
0
0
1
2
3
ErbB4 (μg/ml)

Figure 4.10 Soluble ErbB4 protein blocks neuronal surface survival activity

Soluble ErbB4 protein was also used in neuron-Schwann cell precursor co-cultures to block the survival activity generated by neuronal surface molecules. Almost 60% of survival activity is blocked by 9μg/ml of ErbB4 in a 20 hr assay.

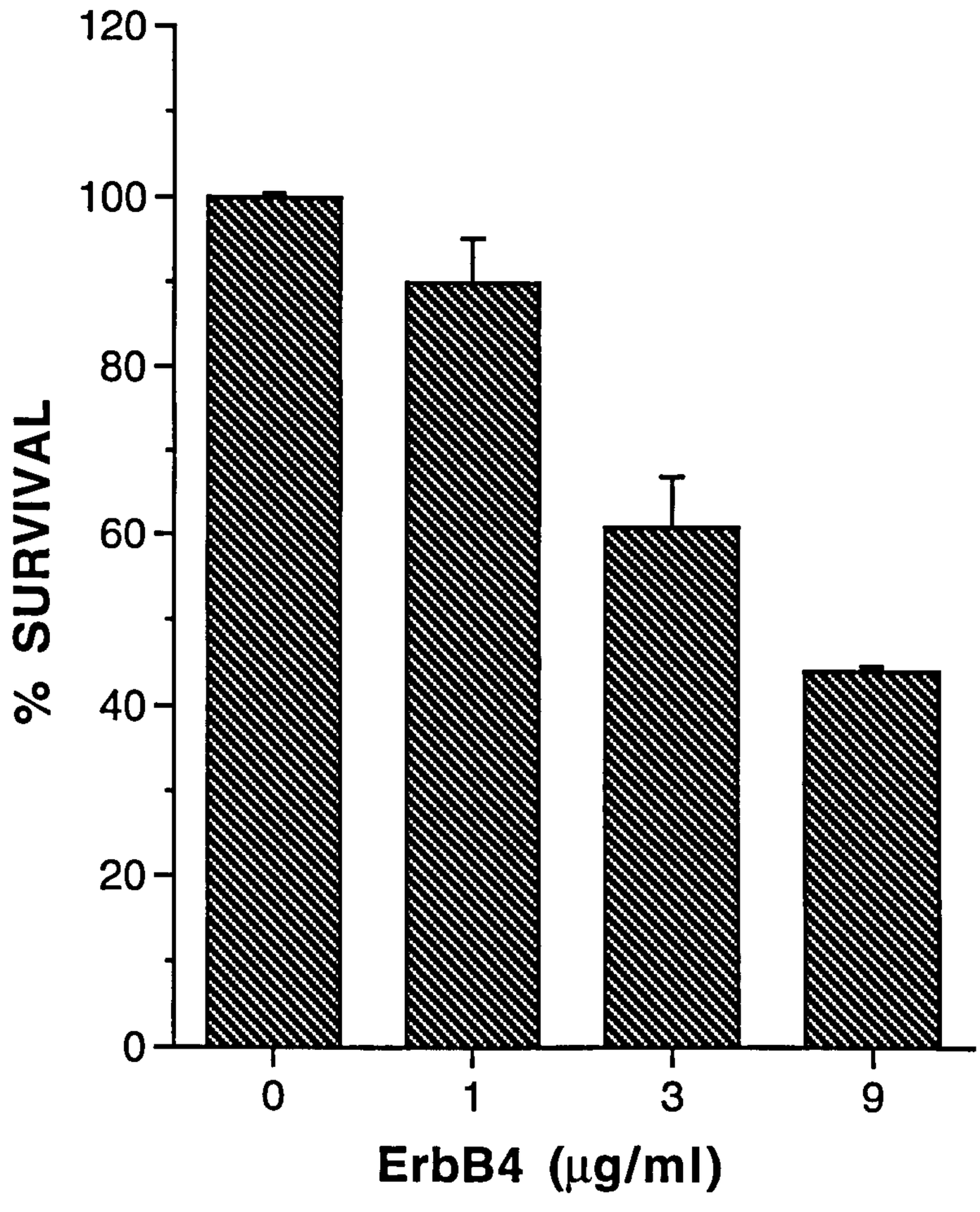
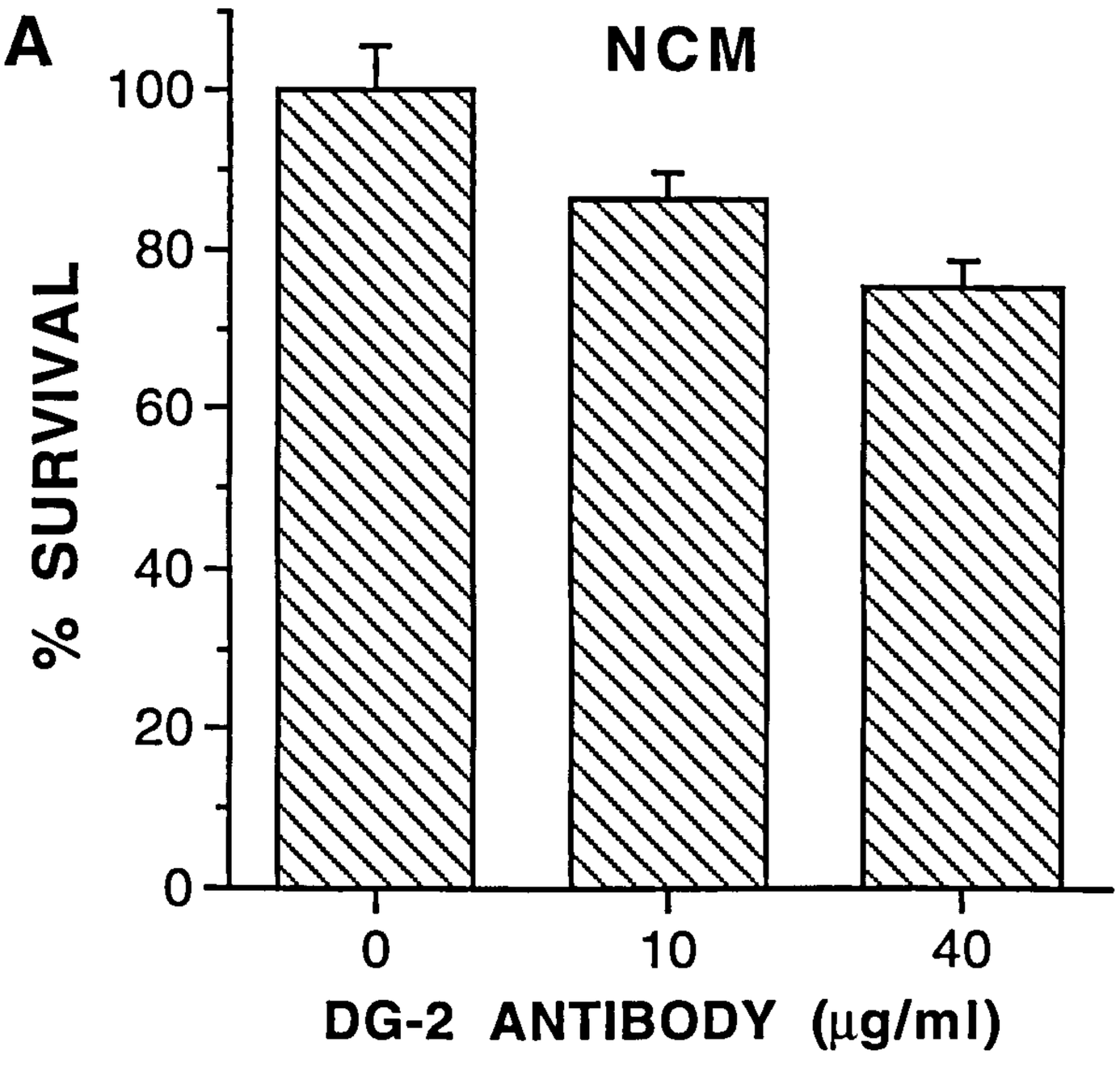

120
100
80
60
40
20
0
% SURVIVAL
0
1
3
9
ErbB4 (μg/ml)

Figure 4.11 The survival activity of FGF-2 in neuron conditioned medium

An FGF-2 monoclonal antibody DG-2 blocks only 25% of survival activity in neuron conditioned medium (A), while the same antibody blocks almost 90% survival activity in FGF-2 containing medium (B). The survival assay was carried out on E14 Schwann cell precursors in a 20 hr assay as shown before.


A
NCM
100
80
60
40
20
0
% SURVIVAL
0
10
40
DG-2 ANTIBODY (μg/ml)

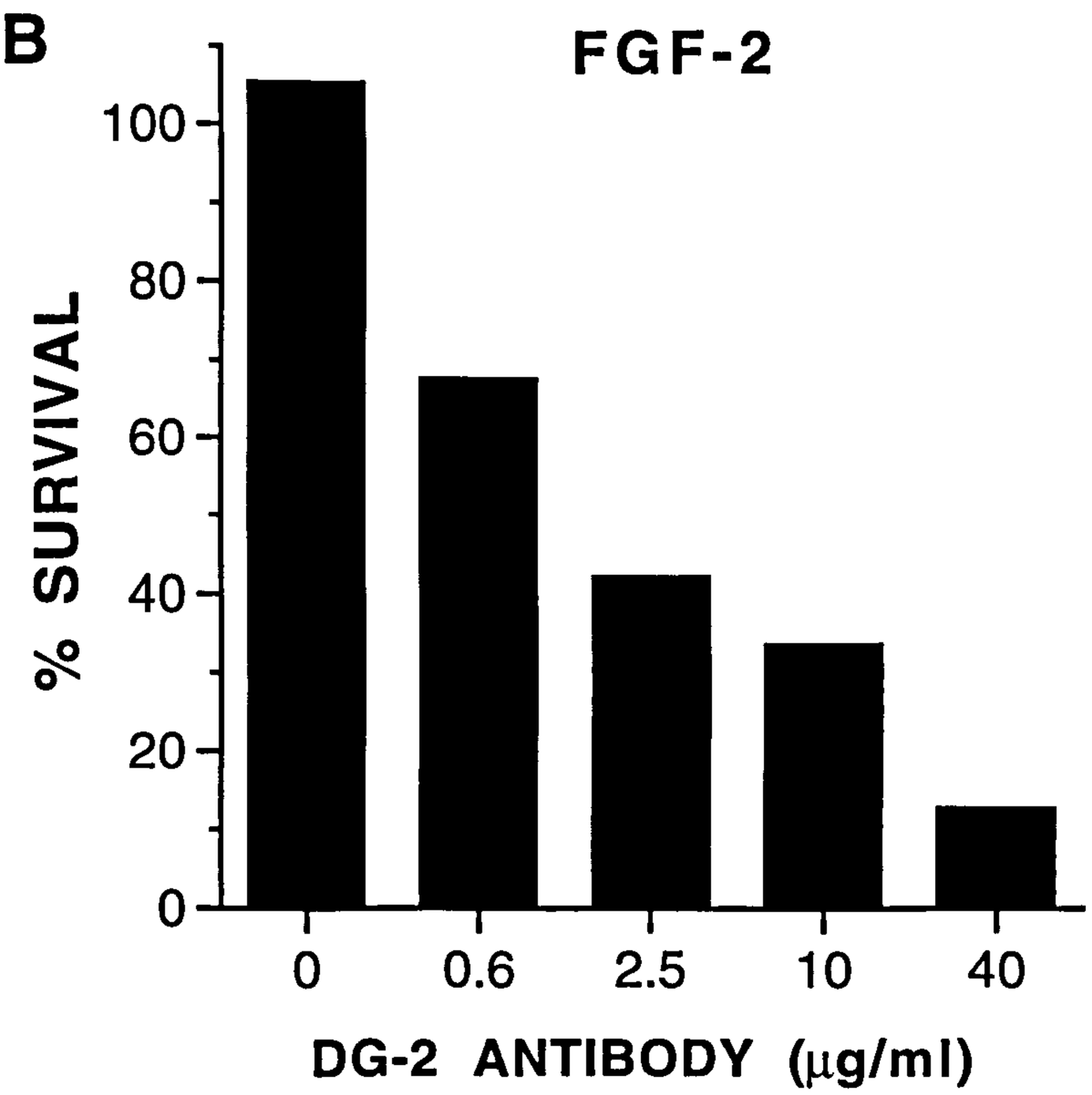

B
FGF-2
100
80
60
40
20
0
% SURVIVAL
0
0.6
2.5
10
40
DG-2 ANTIBODY (μg/ml)

Figure 4.12 DNA synthesis of Schwann cell precursors in the neuron-Schwann cell precursor co-cultures

E14 Schwann cell precursors were co-cultured with pure DRG neurons for 1-14 days. The DNA synthesis of precursors was monitored by introducing a 1.5 hr BrdU pulse on each culturing day. The results show that DNA synthesis of the precursors in co-culture is similar to the DNA synthesis seen in developing nerve (Figure 1.2).

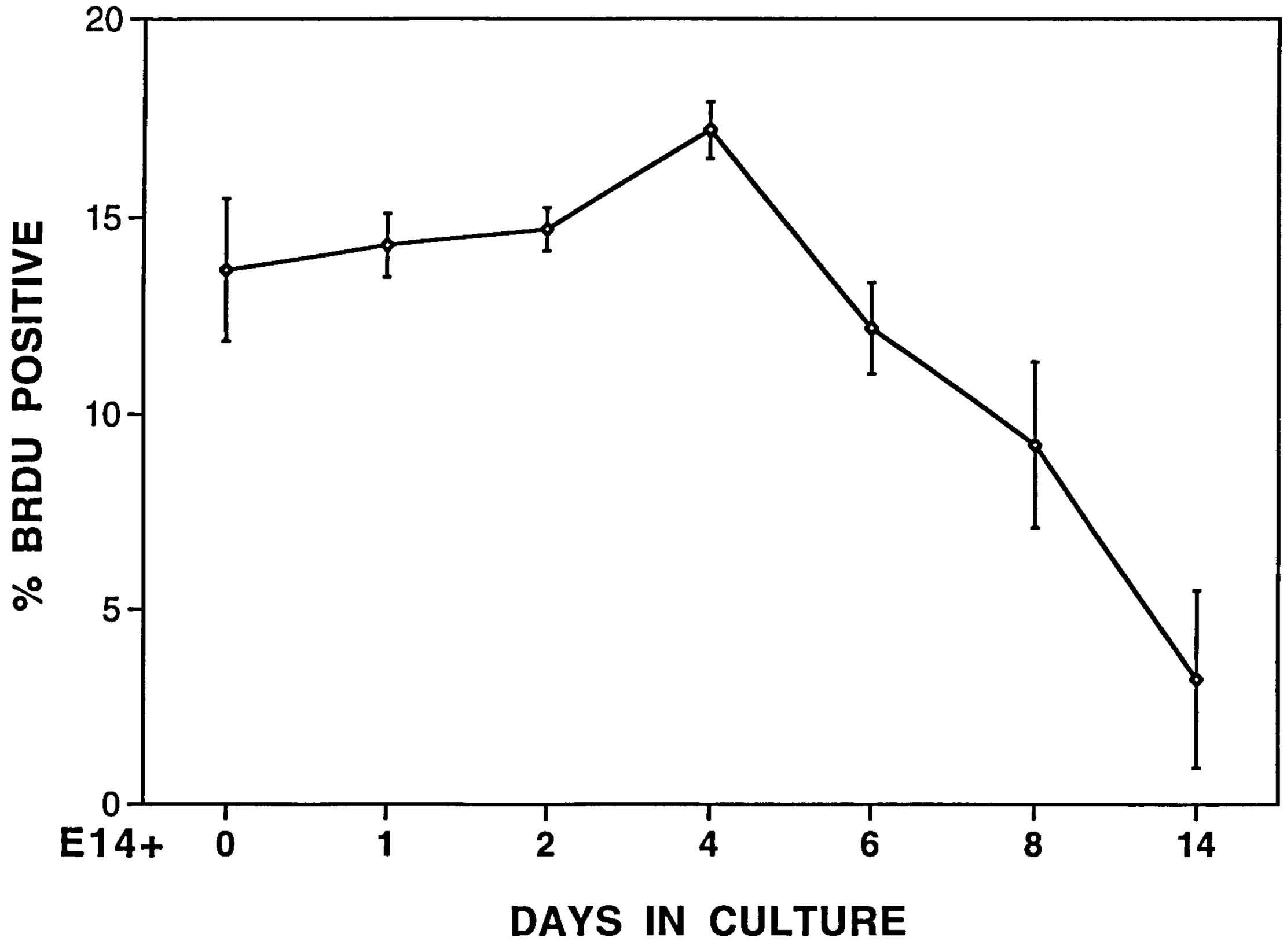

20
15
10
5
0
% BRDU POSITIVE
E14+
0
1
2
4
6
8
14
DAYS IN CULTURE

Figure 4.13 Neurons stimulate Schwann cell precursor DNA synthesis

Schwann cell precursors were co-cultured with pure DRG neurons for 4 days, precursors in contact with neurons and neurites were stimulated to synthesize DNA. (A) BrdU positive-nuclei in precursors labelled with L1 antibody. (B) neuronal cell body and neurites were labelled by CFSE. (C) phase-contrast view. Magnification 700X

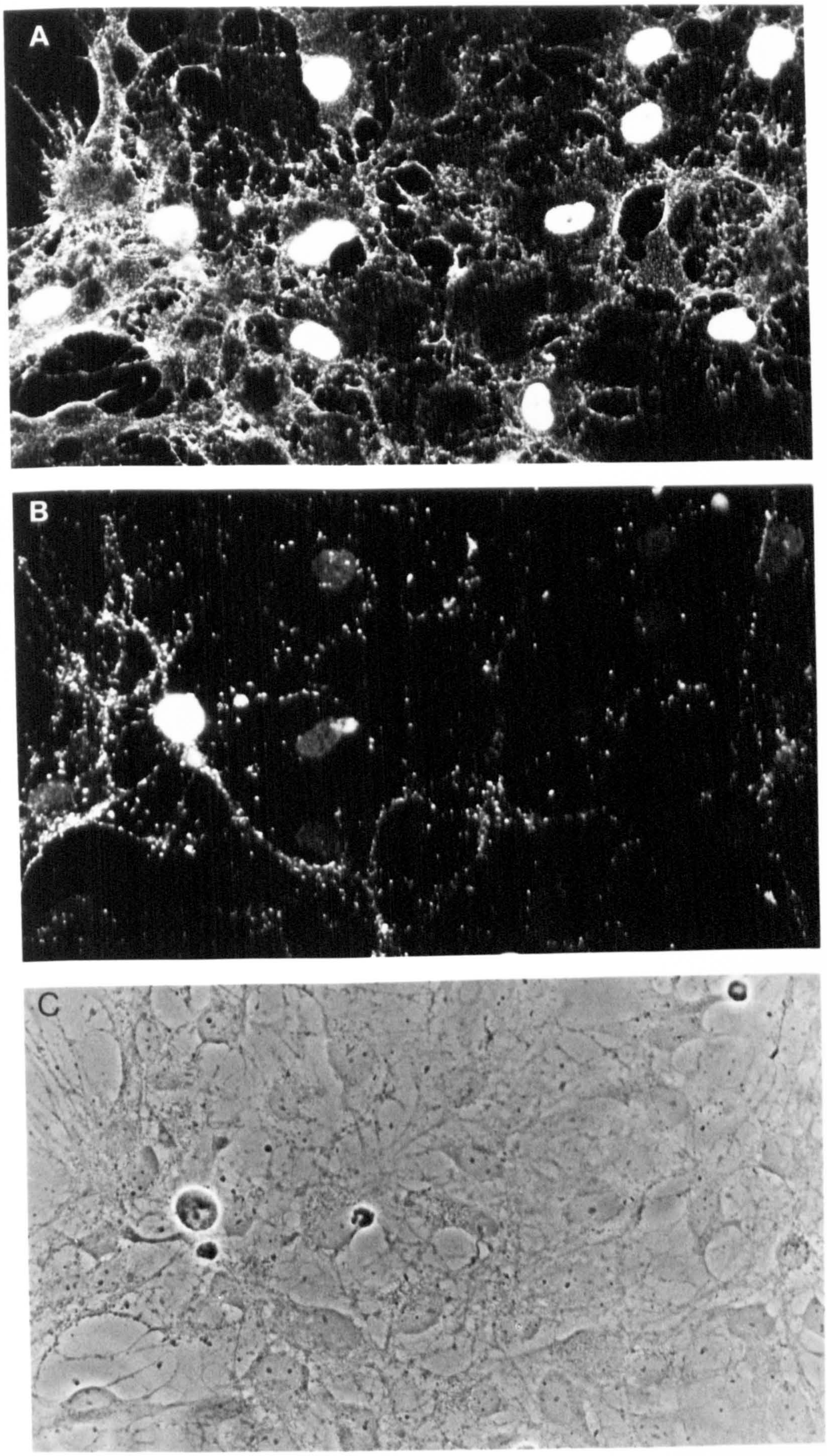
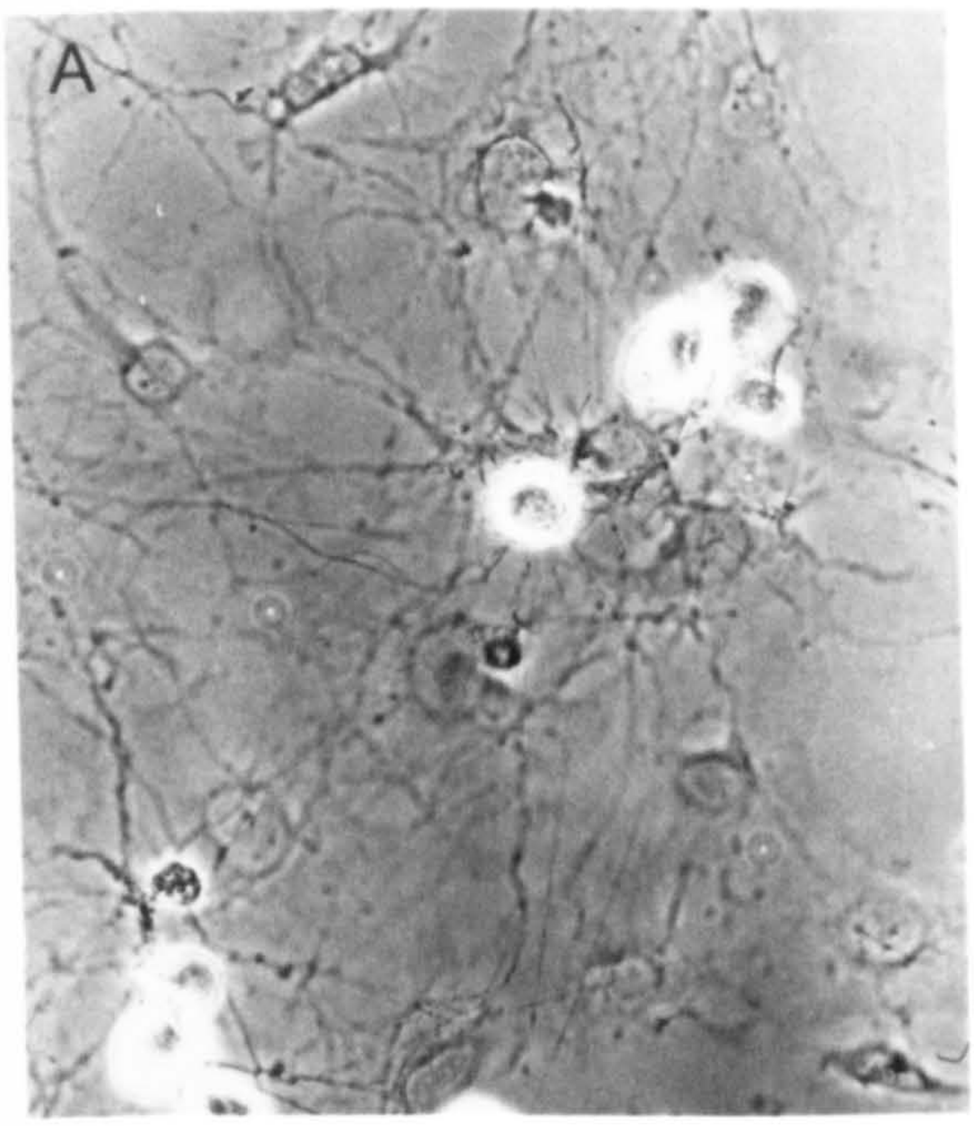
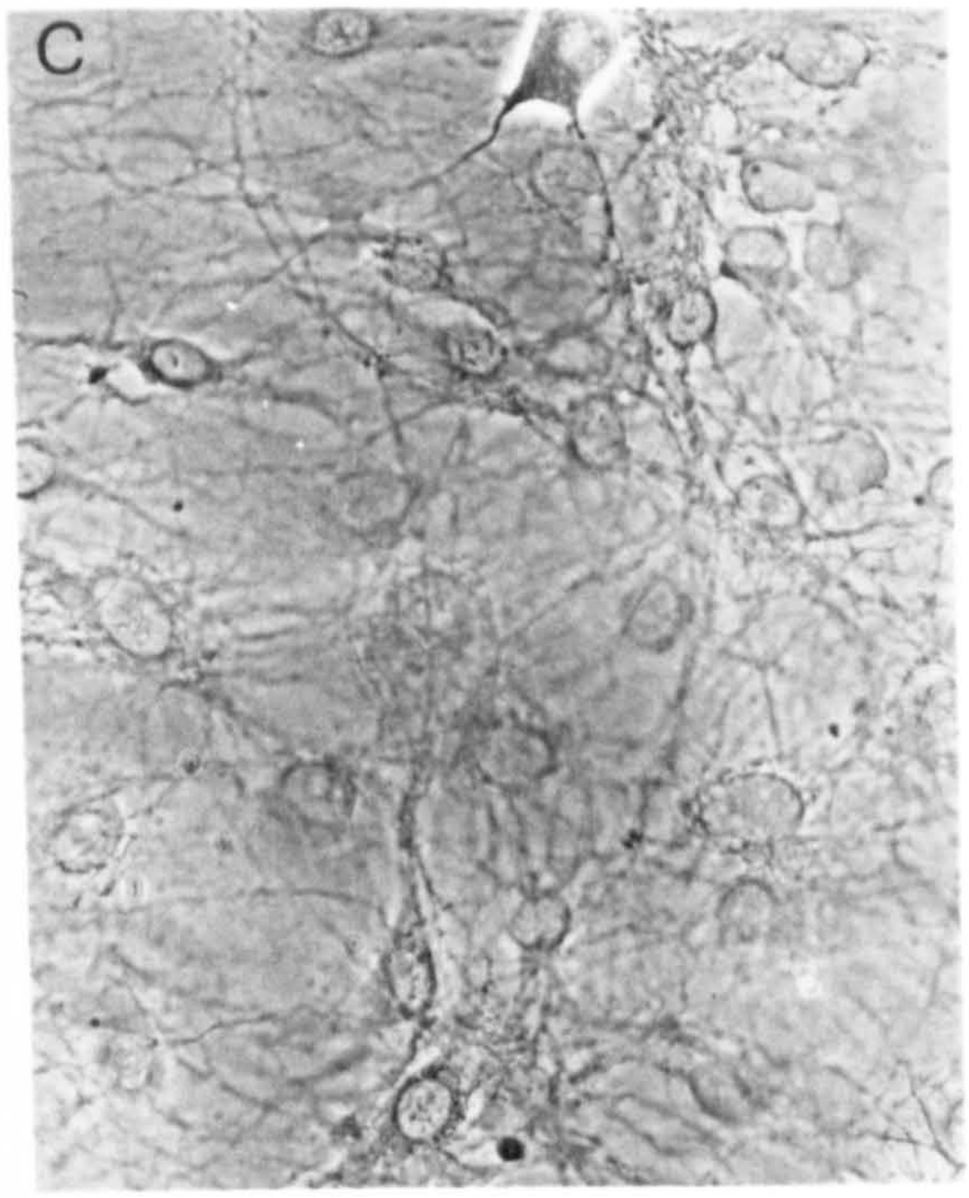

A
B
C

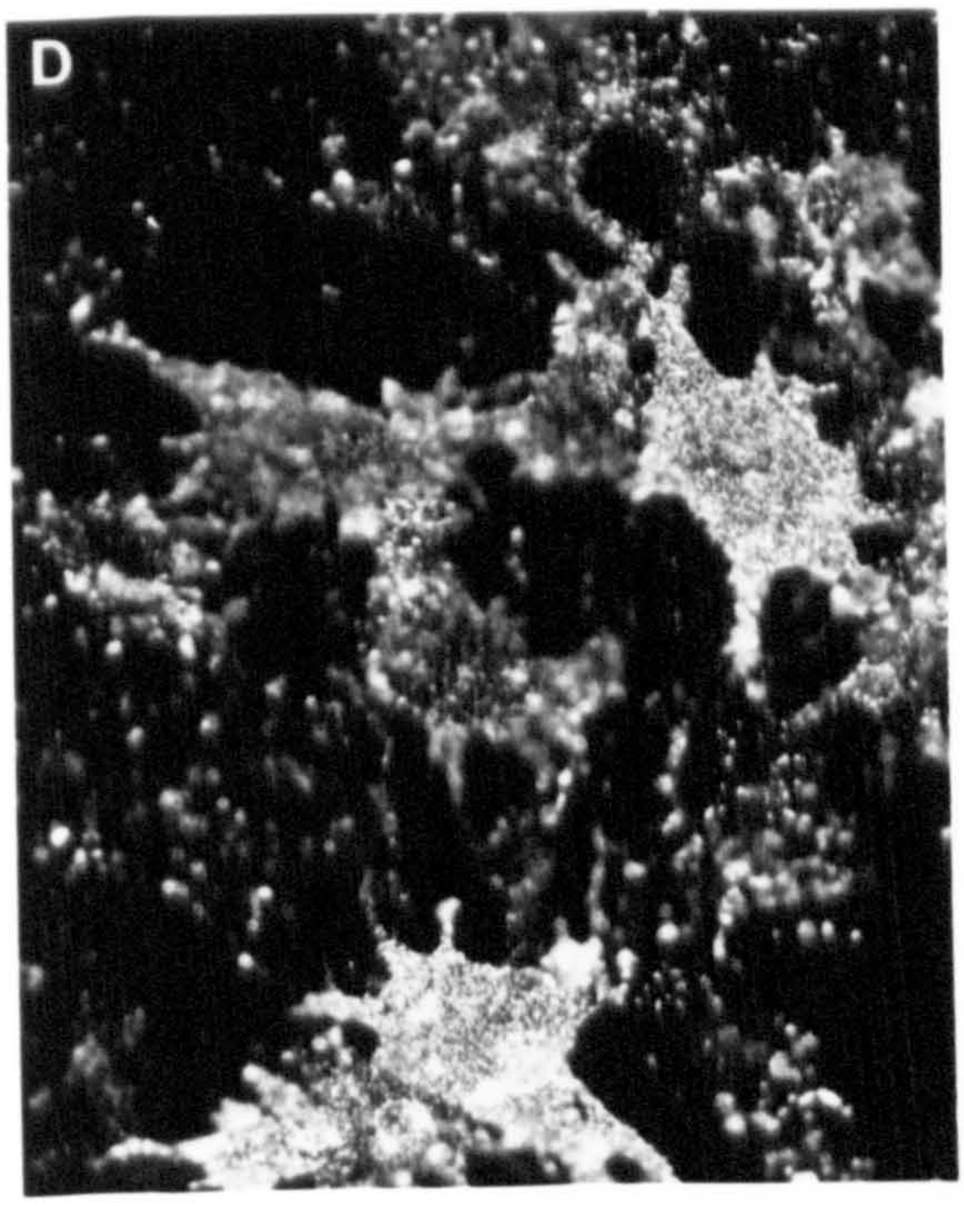
Figure 4.14 Neurons regulate Po expression in Schwann cell precursors

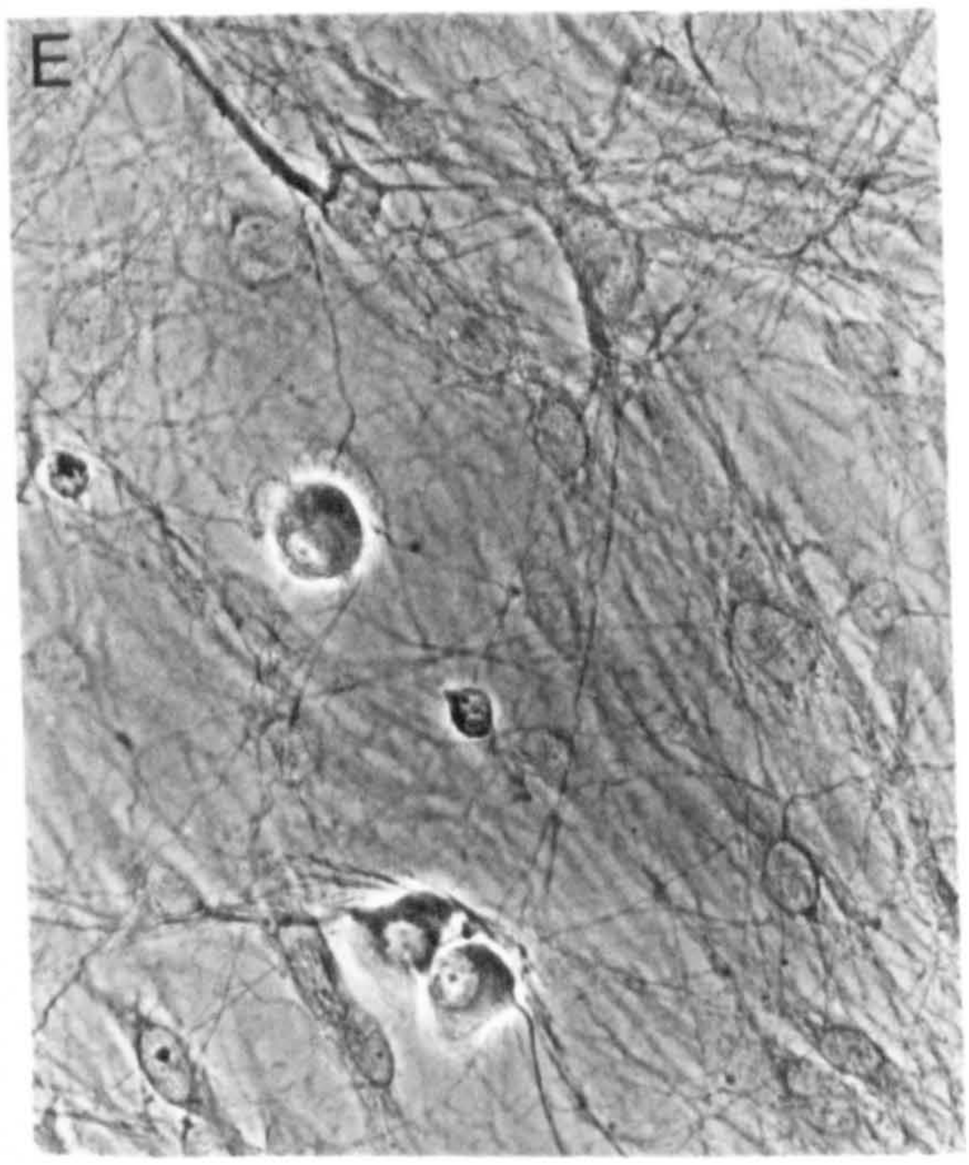
Schwann cell precursors were co-cultured with purified neurons for 2-7 days. Po antibody was used to label the Po positive cells. The results show that at day 2, precursors are induced to express low levels of Po (B). At the day 4, the Po levels in individual cell are elevated (D), and at day 7 cells tend to be much more bi-polar and the Po levels in cells are even higher (F). A, C, E are the corresponding phase-contrast views. Magnification 600X


A


B


C


D


E

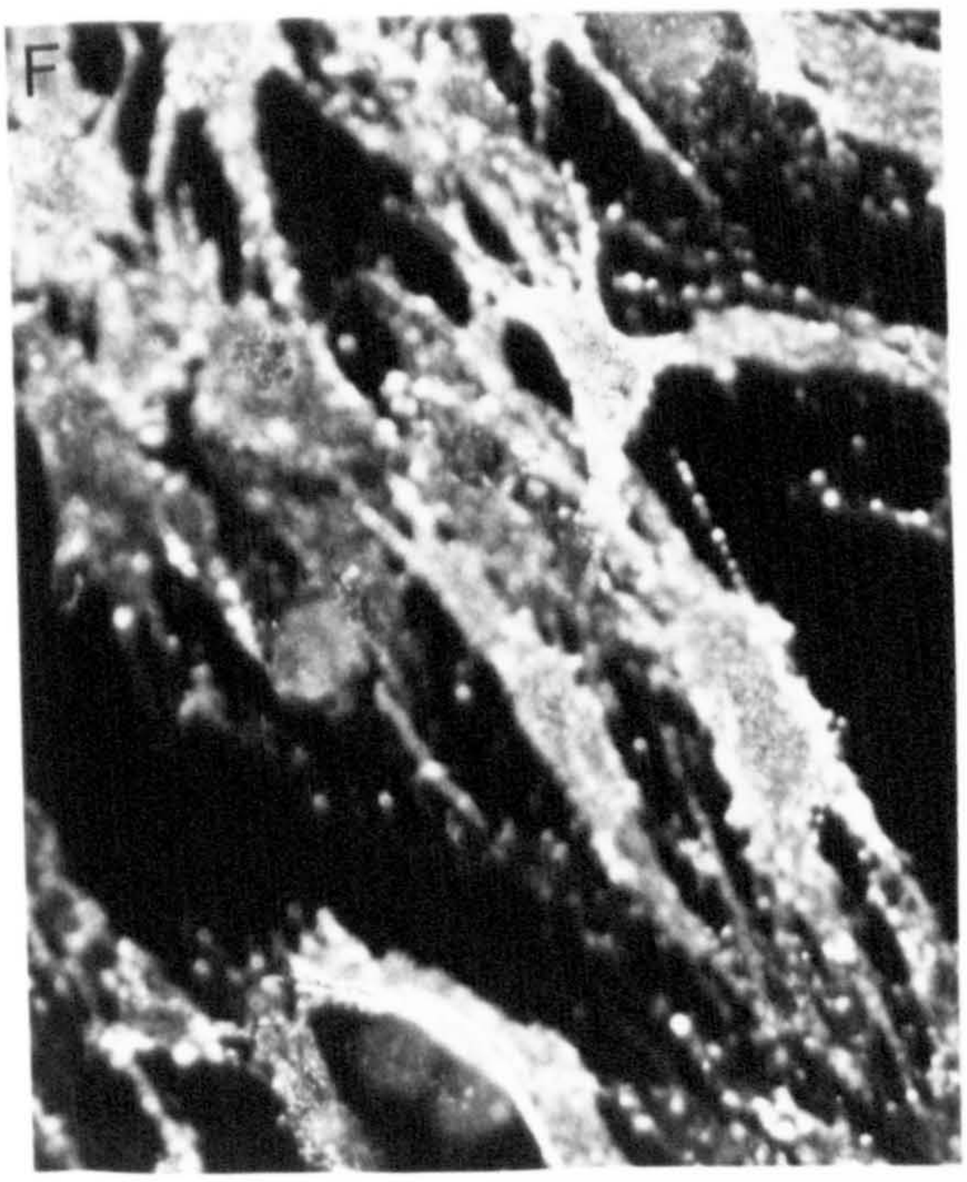
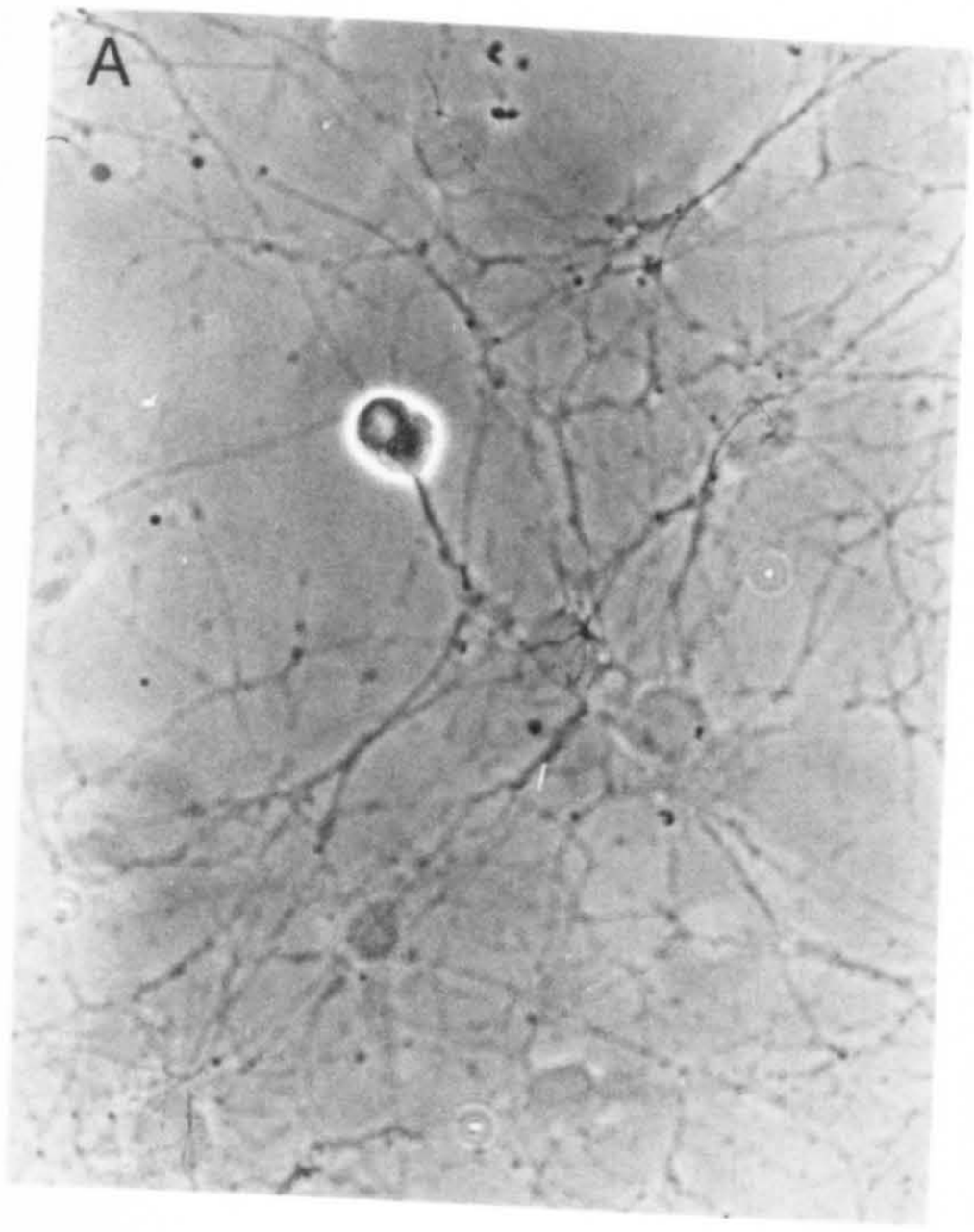
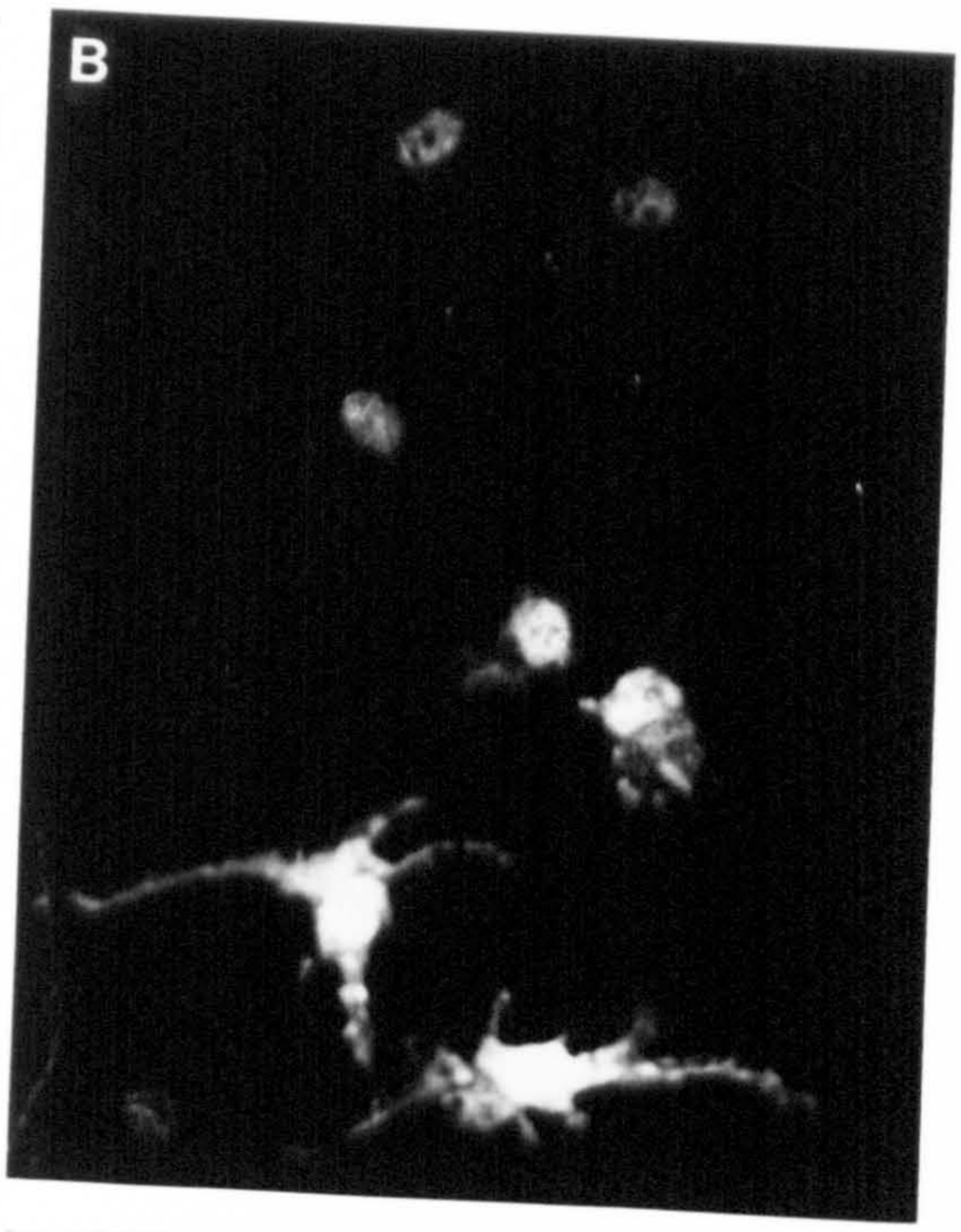
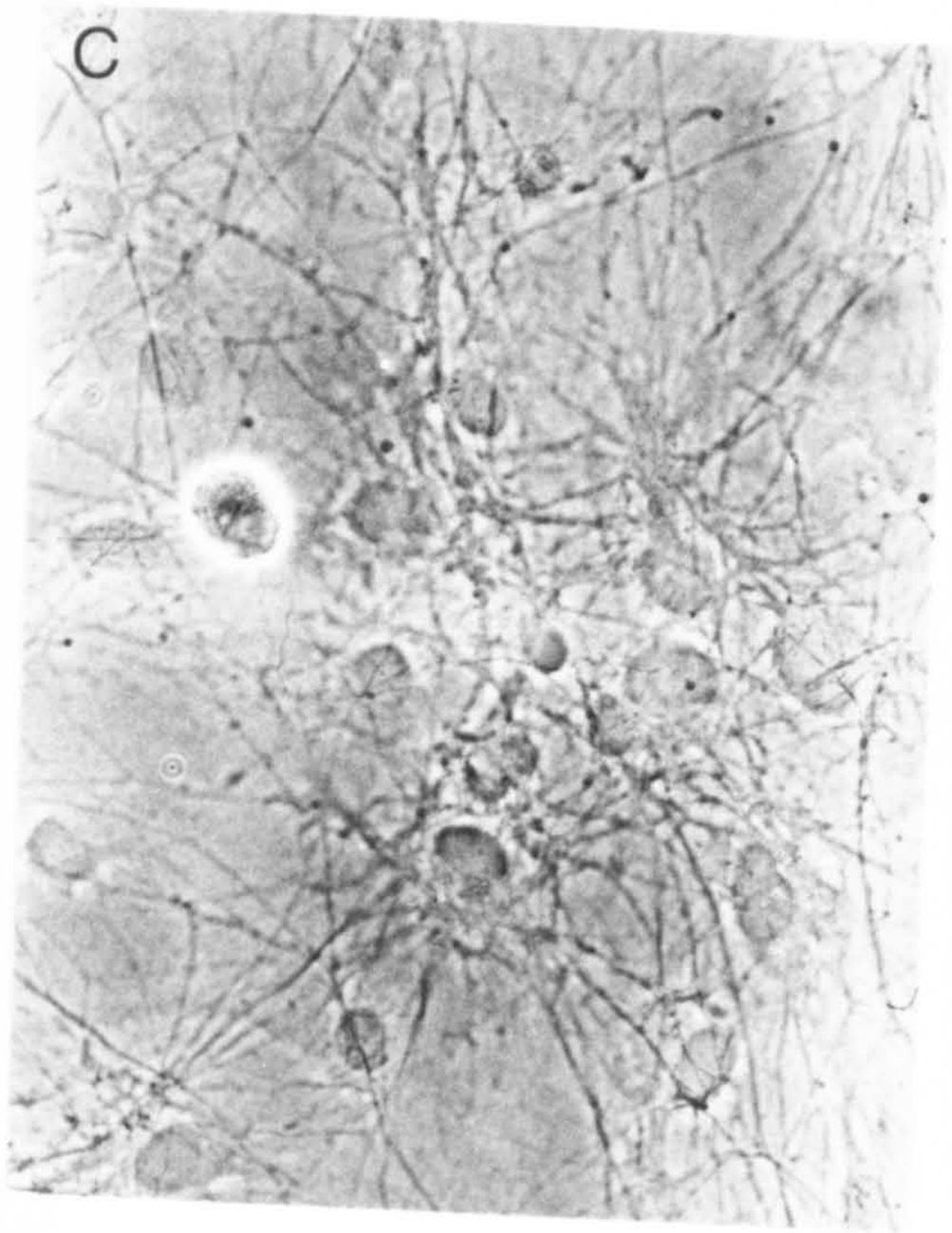

F

Figure 4.15 Neurons induce S100 expression in Schwann cell precursors

Schwann cell precursors were co-cultured with purified neurons. Anti S-100 antibody was used on the 2nd and 4th day of co-culture. The results show that only a few cells are S-100 positive at day 2 (A,B), and almost all cells are S-100 positive at day 4 (C,D). Magnification 600X


A

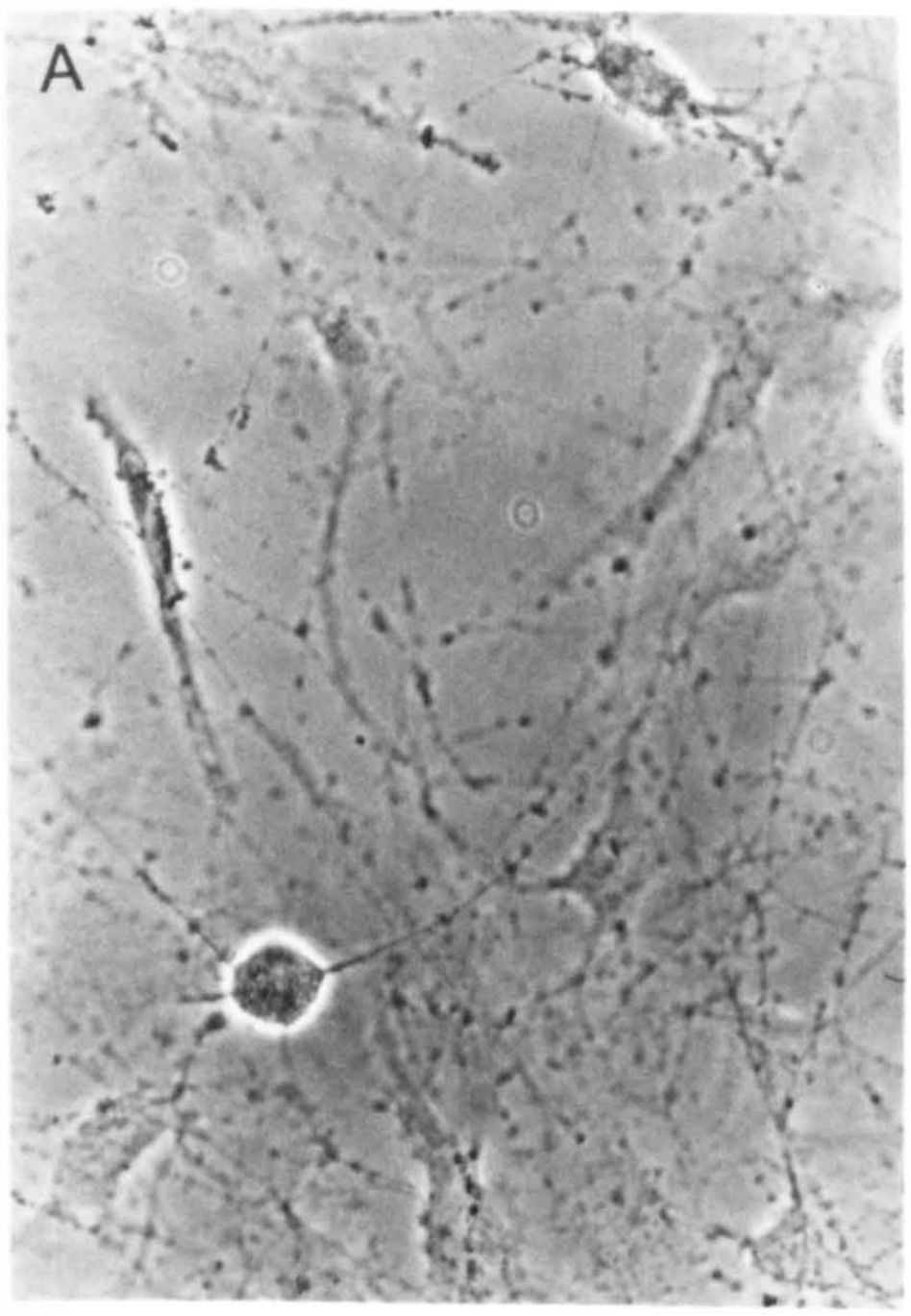

B

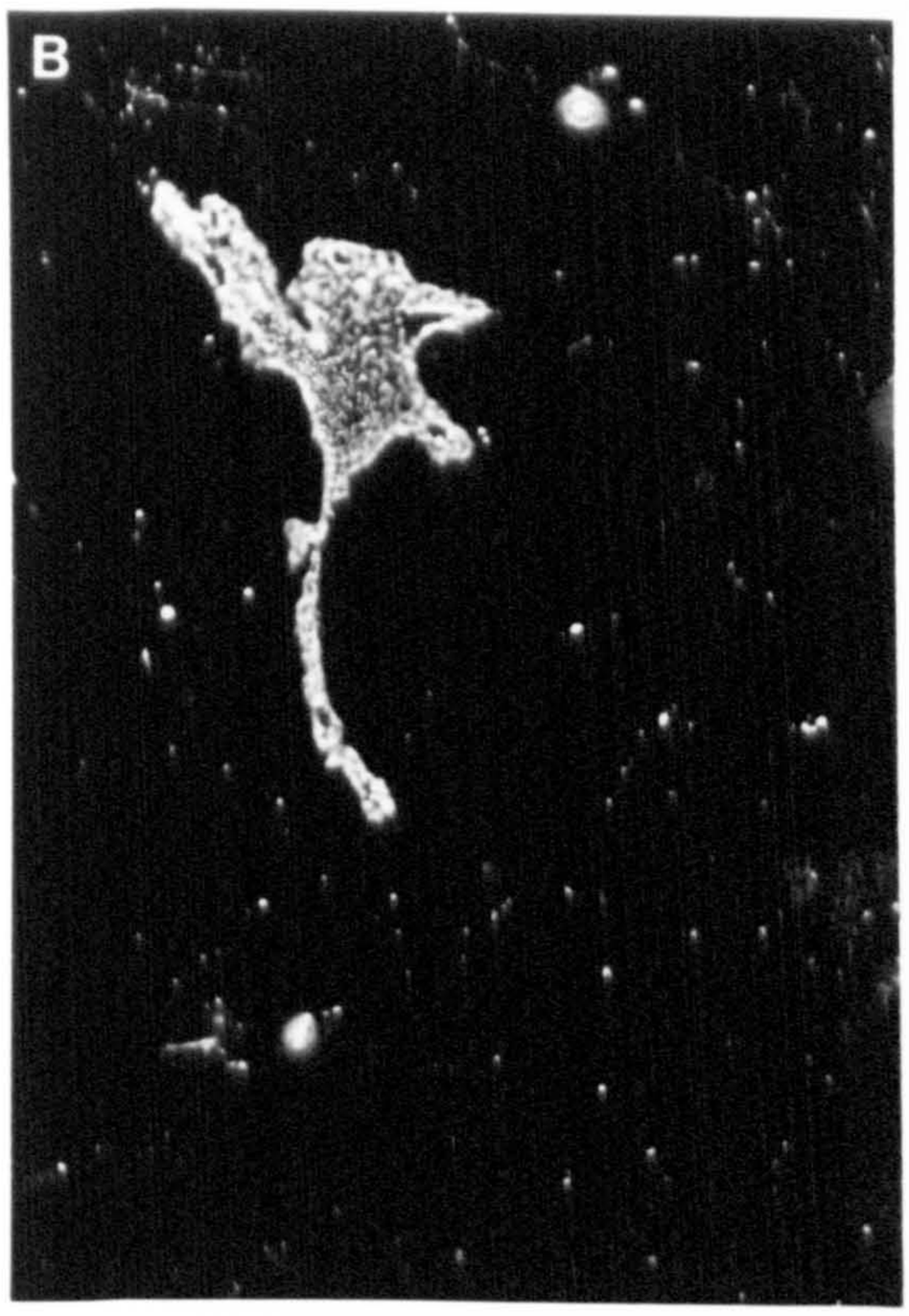

C

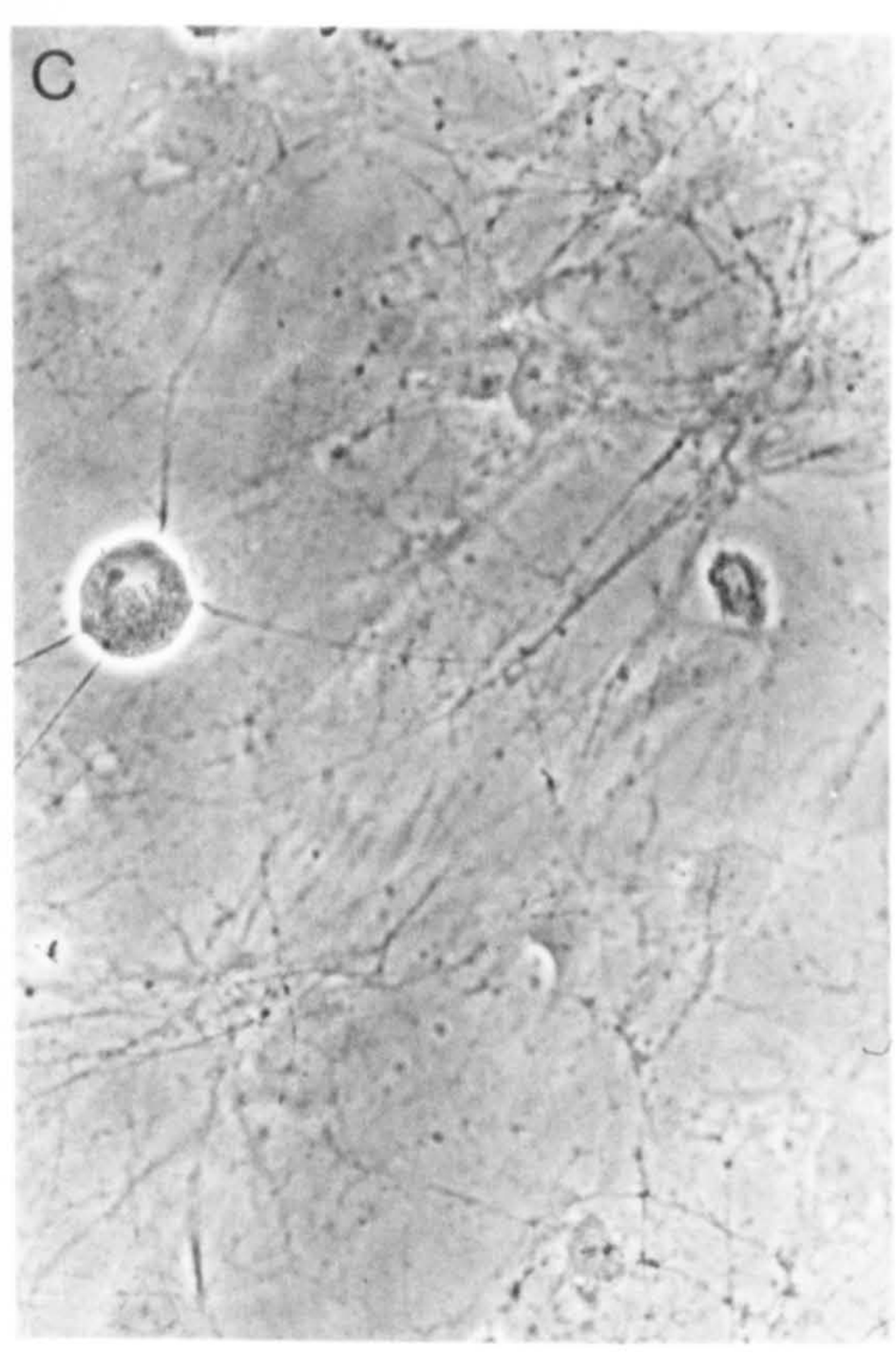

D

Figure 4.16 Neurons induce 04 expression in Schwann cell precursors

Schwann cell precursors were co-cultured with purified neurons. 04 antibody was used on the 2nd and 4th day of co-culture. The results show that at day 2, less than 10% of the cells are 04 positive (A,B), and most cells are 04 positive at day 4 (C,D). Magnification 600X


A


B


C

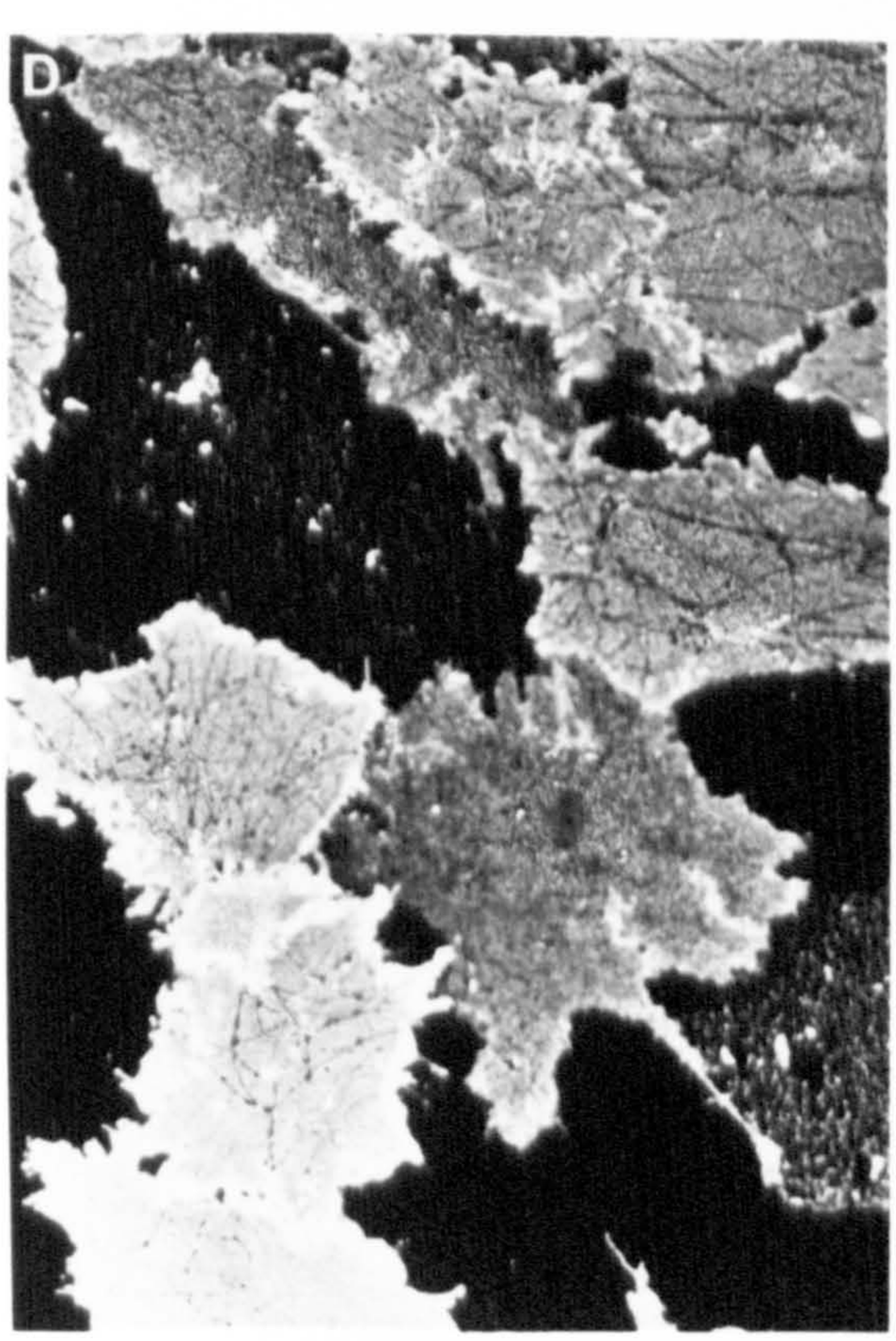
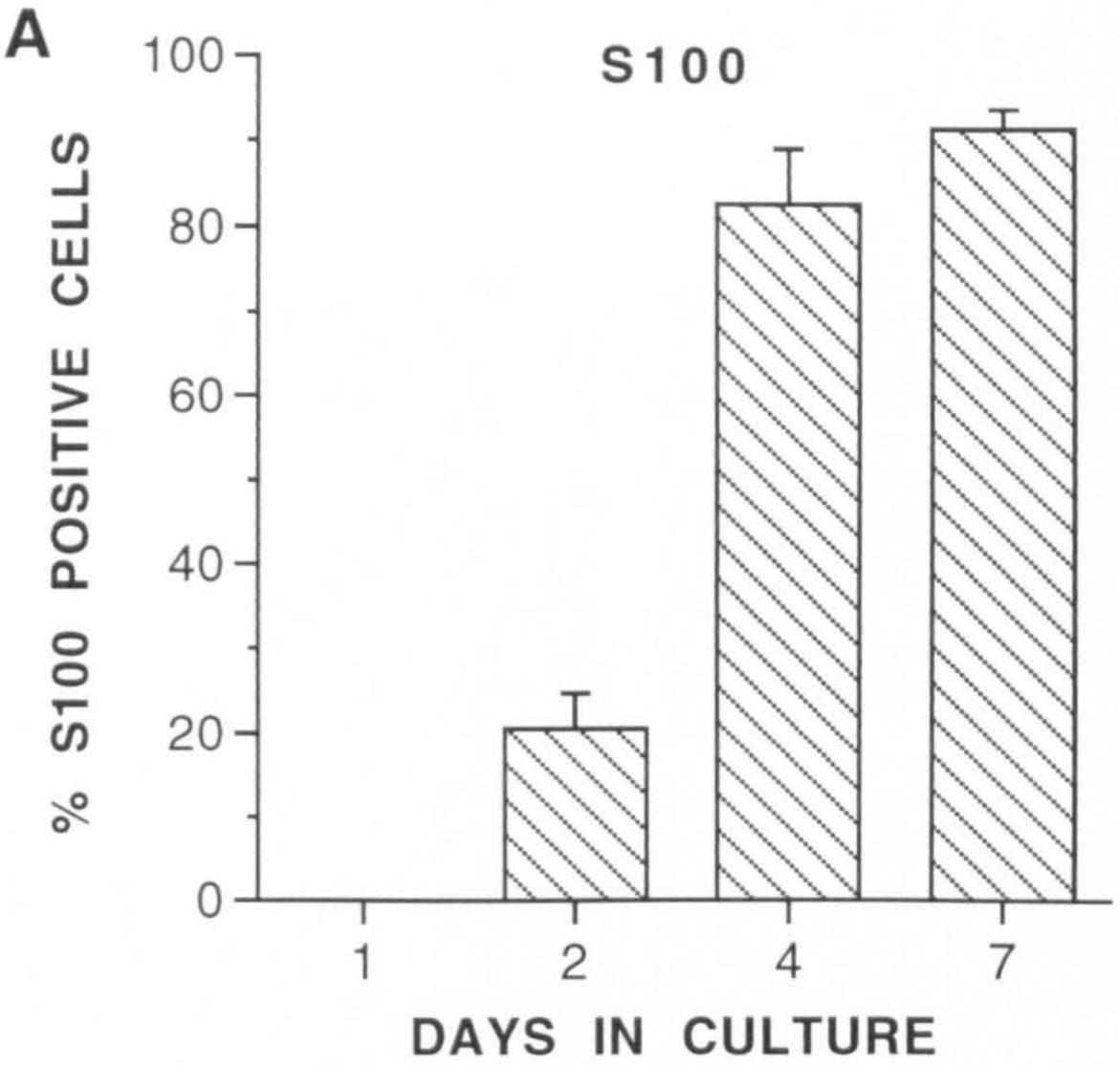

D

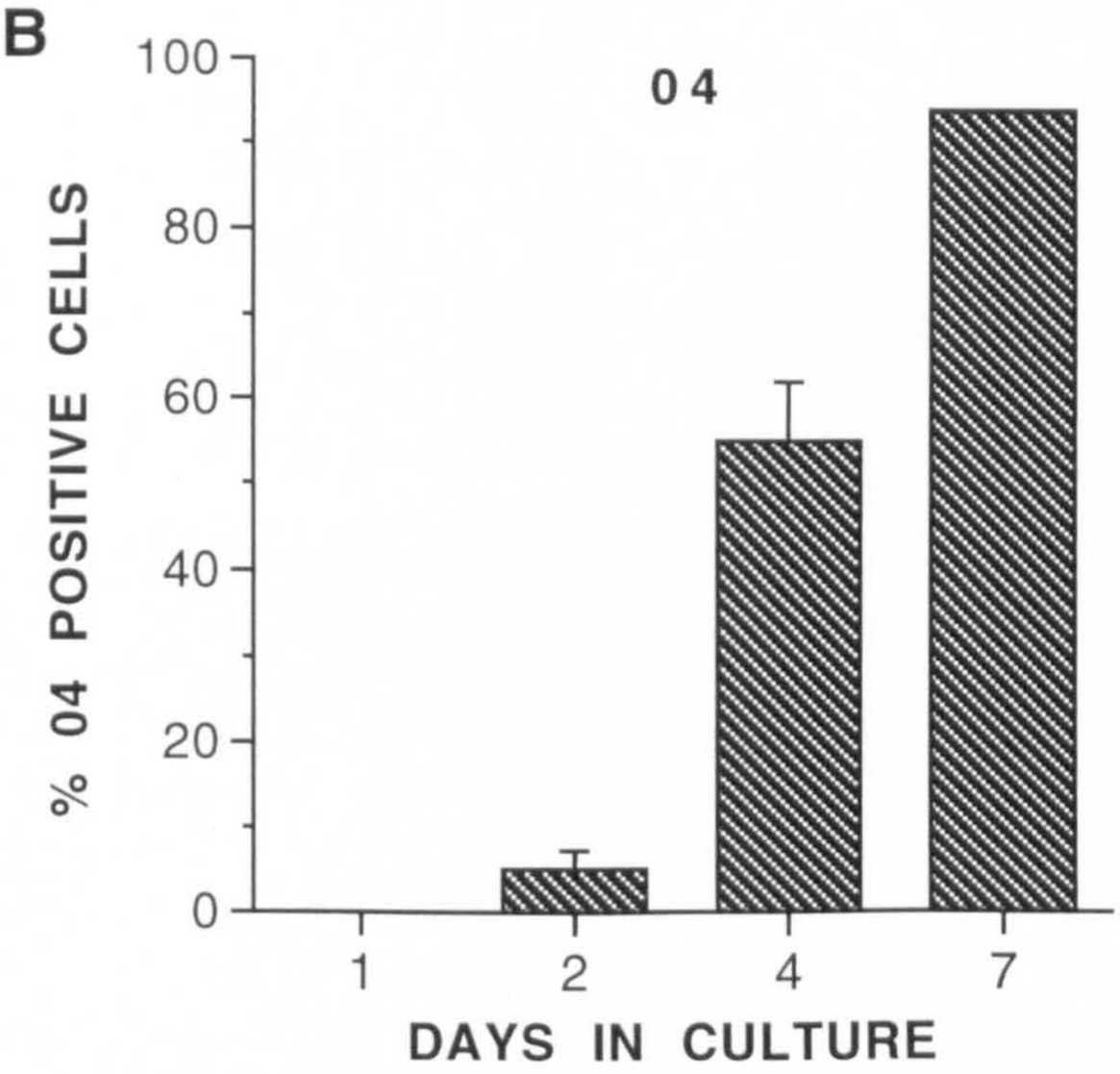
Figure 4.17 S100, 04 and Po expression of the cells in neuron-Schwann cell precursor co-cultures

Schwann cell precursors were co-cultured with purified neurons for 1-7 days. S-100 or 04 or Po antibodies were used to label the cells at day 1, day 2, day 4 and day 7. The results show that at day 1, no S-100, 04 and Po positive cells are seen, S-100 positive cells are increased from 20% at day 2 to more than 80% at day 4 (A), and 04 positive cells are increased from less than 10% at day 2 to 90% at day 7 (B). The Po positive cells are decreased from day 2 to day 7 (C), but the Po levels in individual cells are increased (Figure 4.14)

A


S100
% S100 POSITIVE CELLS
100
80
60
40
20
0
1
2
4
7
DAYS IN CULTURE

B


04
% 04 POSITIVE CELLS
100
80
60
40
20
0
1
2
4
7
DAYS IN CULTURE

C

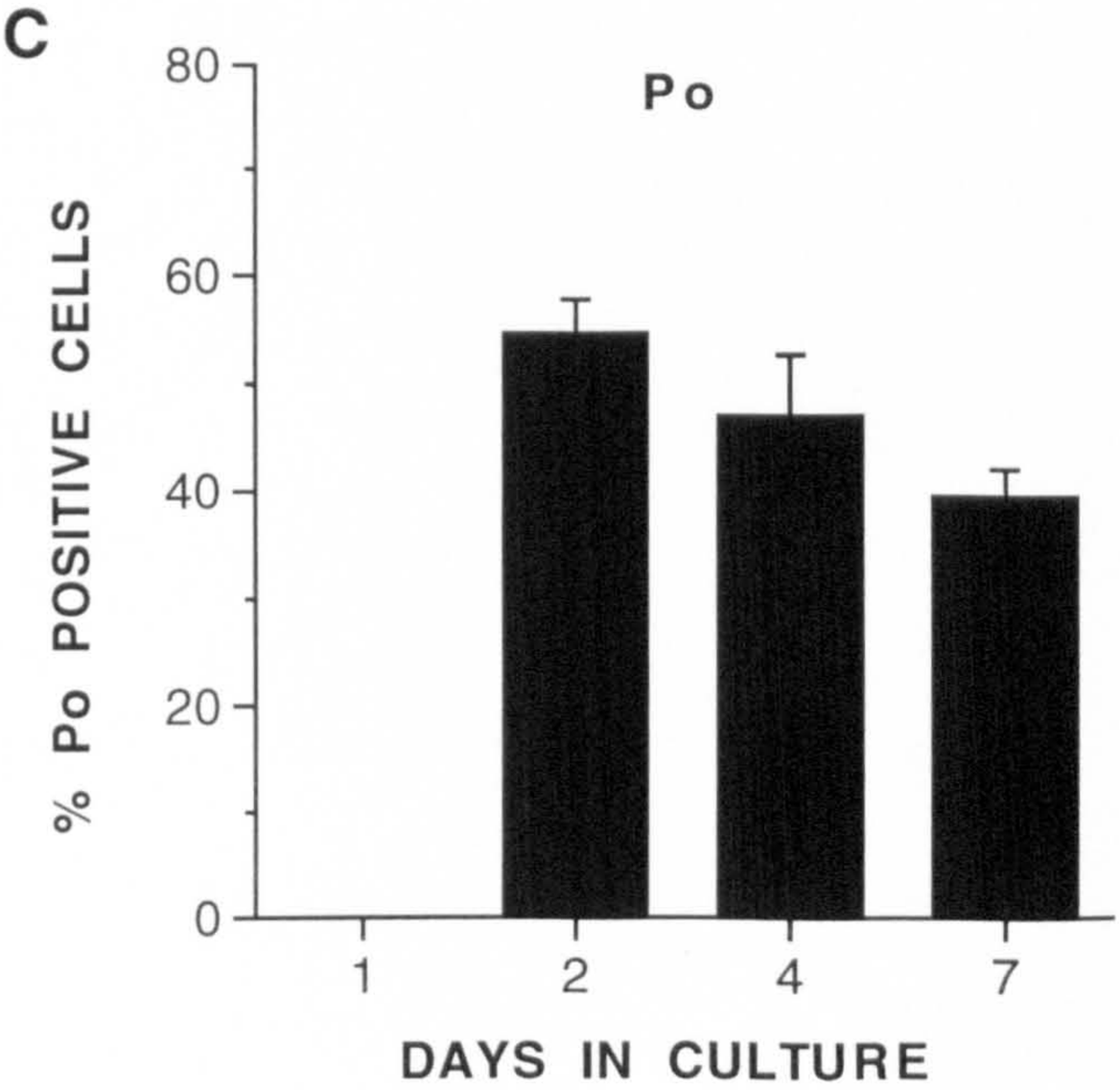

Po
% Po POSITIVE CELLS
80
60
40
20
0
1
2
4
7
DAYS IN CULTURE

Figure 4.18 Neurons induce Schwann cell precursors to express Po mRNA.

Schwann cell precursors co-cultured with purified neurons were induced to express Po mRNA. In situ hybridisation was carried out by using digoxigenin-labelled probes. The results show that at day 1, no Po mRNA was seen (B), and at day 7 cells were heavily labeled (D). A and C are the corresponding phase-contrast views. Magnification 600X

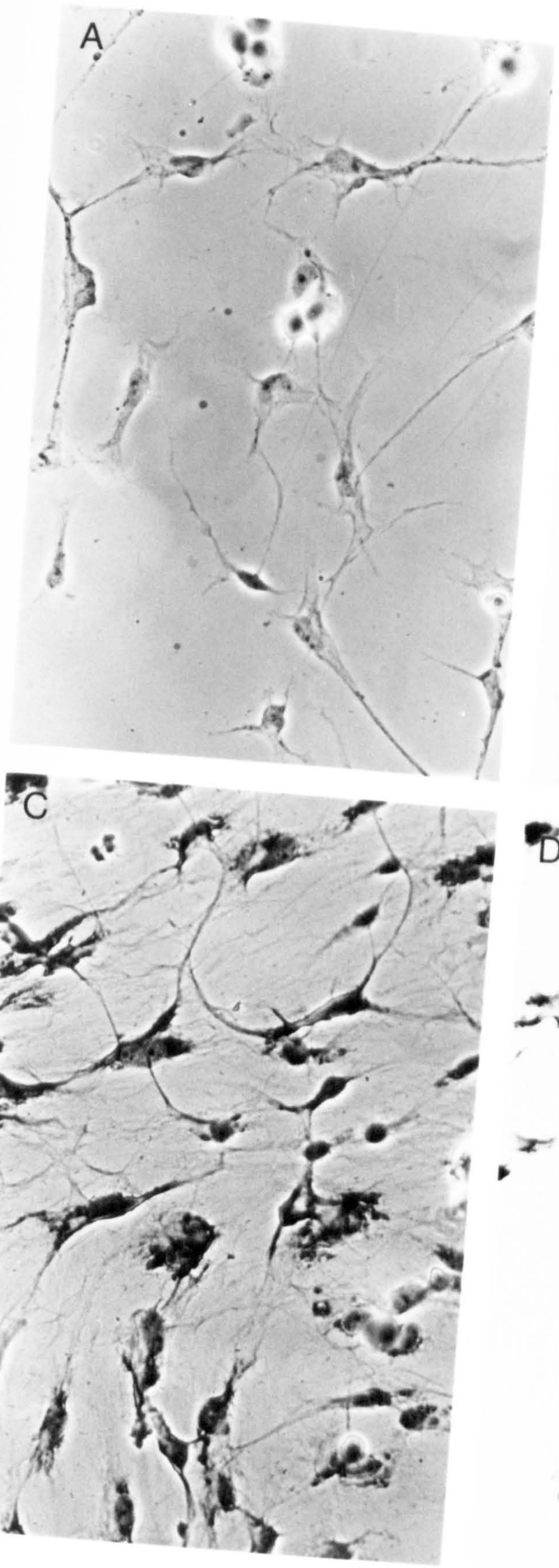
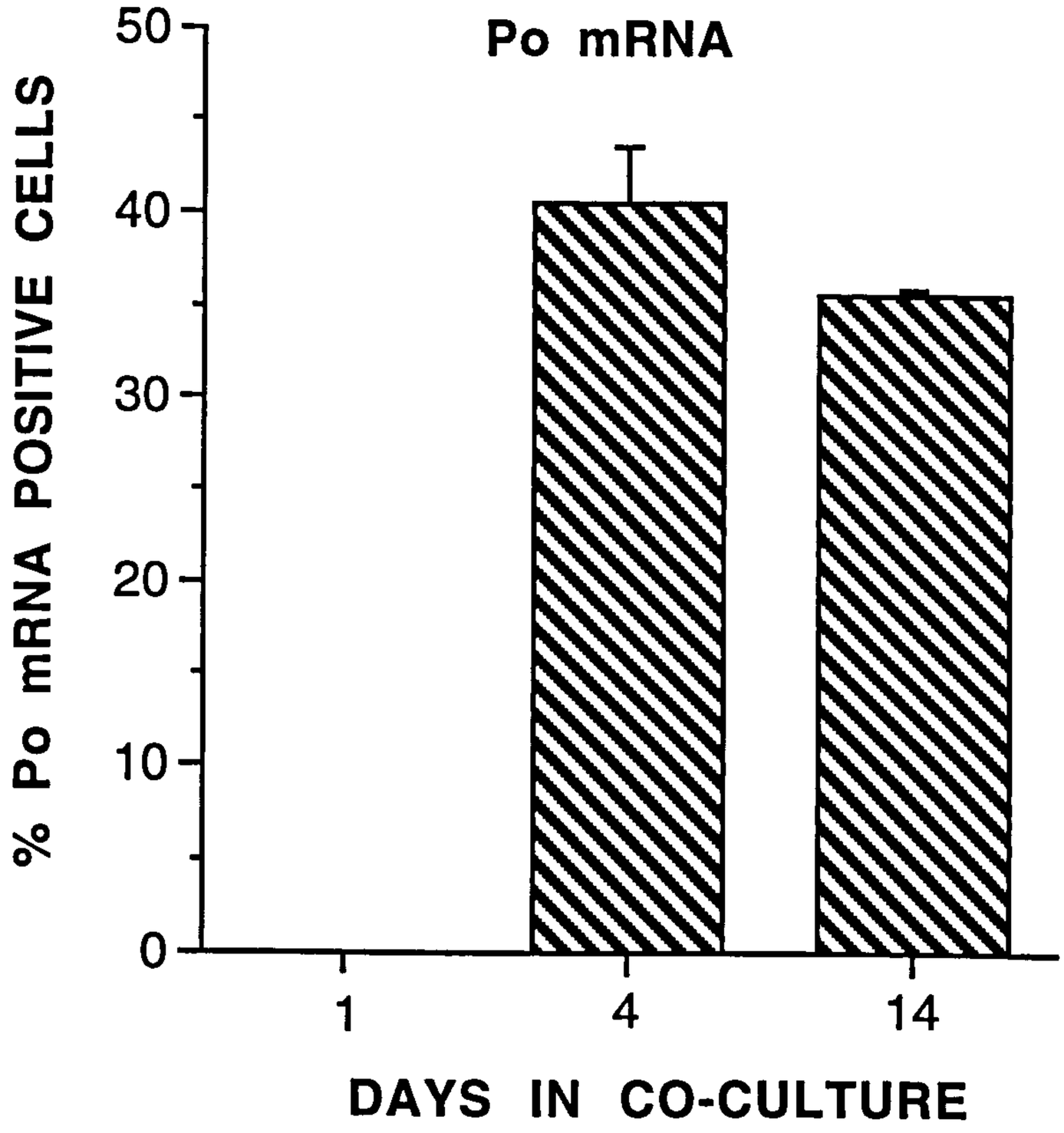

A
B
C
D

Figure 4.19 Po mRNA expression of the cells in neuron-Schwann cell precursor co-cultures

Schwann cell precursors were co-cultured with purified neurons for 1-14 days. Dogoxigenin-labeled Po probes were used to detect the Po mRNA in cells. The results show that the percentage of Po mRNA positive cells is increased at day 4 and slightly decreased at day 14.


Po mRNA
% Po mRNA POSITIVE CELLS
50
40
30
20
10
0
1
4
14
DAYS IN CO-CULTURE

CHAPTER 5

MITOGENIC RESPONSES IN CELLS OF THE SCHWANN CELL LINEAGE

Introduction

The regulation of Schwann cell proliferation is an important part of peripheral nerve development and Wallerian degeneration. In the early stages of rat development, Schwann cell precursors, which are generated from neural crest undergo significant DNA synthesis in vivo (Stewart et al., 1993). The cells continue to proliferate during Schwann cell precursor maturation and throughout early Schwann cell development with a peak at E19-E20. After birth, DNA synthesis of the Schwann cells declines rapidly, coinciding with the appearance of myelin. In adult rats, Schwann cells are generally quiescent in normal peripheral nerve (Friede and Samorajski, 1968; Komiyama and Suzuki, 1991; Stewart et al., 1993).

If an adult nerve is transected, however, the Schwann cells in the distal stump of the nerve proliferate vigorously 3 days after the injury (Abercrombie and Johnson, 1946; Clemence et al., 1989; Komiyama and Suzuki, 1992). The proliferation rate remains elevated up to 8 days after transection, and both myelin-forming and non-myelin-forming Schwann cells contribute to this massive proliferation (Clemence et al, 1989; Komiyama and Suzuki, 1992). In contrast, transecting the sciatic nerve in a neonatal animal results in a reduction of Schwann cell DNA synthesis (Komiyama and Suzuki, 1992). It has been suggested that myelin debris is an important source of the mitogen which induces adult Schwann cell division in degenerating nerves, since myelin-enriched membrane fractions and myelin basic protein are able to stimulate DNA synthesis in neonatal Schwann cells in culture (Yoshino et al., 1978; Baichwal and DeVries, 1989). Furthermore, invading macrophages have been shown to digest myelin membrane fragments resulting in the production of a Schwann cell mitogen (Baichwal et al., 1989). This concentration on the contribution of extrinsic factors to the mitogenic response during Wallerian degeneration has not been matched by consideration of the role that alteration in the Schwann cells themselves might play. This issue will be addressed in the present chapter.

The technique of Schwann cell culture has been established for nearly a century. The recent purification of Schwann cells most commonly involves culturing cells in serum containing medium for several days or weeks and exposure to anti-mitotic agents (Wood and Bunge, 1975, Brockes et al., 1978). These cultured Schwann cells have been widely used to study the proliferation and differentiation of these cells and also their molecular interaction with neurons (Ratner, 1986; Mirsky and Jessen, 1990). It is clear that Schwann cells generated by this purification method are still myelinogenic in response to axons and the degree to which they respond by DNA synthesis to various recombinant growth factors has been carefully documented (Wood and Bunge, 1975; Wood, 1976; Brockes et al., 1978, Stewart et al., 1991). An issue that generally has not been addressed in these studies, however, is whether the mitogenic response of Schwann cells might be altered by the purification procedure itself. Similarly, insufficient attention has been paid to the possibility that mitogenic responses of Schwann cells might depend on their developmental stage. For these reasons it is unclear how faithfully the data from most current mitogen studies reflect the responses of Schwann cells in developing or denervated nerves in vivo.

It is clear that Schwann cells in short term cultures are quiescent in serum free medium. Even in serum containing medium these cells normally divide very slowly, although this will depend to some extent on type of serum that is used (Raff et al., 1978; Brockes et al., 1980; Porter et al., 1987; Scorpini et al., 1988). When the cells are exposed to some growth factors proliferation can be triggered. Thus, FGF, PDGF-BB, TGFβ and GGF are mitogens for Schwann cells in serum-containing medium and the mitogenic response to these factors is enhanced by forskolin or cholera toxin both of which elevate intracellular cAMP levels (Ridley et al., 1989; Eccleston et al., 1989; Weinmaster and Lemke, 1990; Davis, 1990). In serum free defined medium only GGF and hepatocyte growth factor (HGF) show mitogenic potential when used alone, while the mitogenic activity of FGF and PDGF-BB depends on combination with forskolin or cAMP analogues (Stewart et al., 1991; Hardy et al., 1992; Krasnoselsky et al., 1994). Although TGFβ can replace cAMP in stimulating Schwann cell DNA synthesis when combined with FGF-2, the mitogenic potential generated by this combination is limited (Schubert, 1992). A recent study

indicates that TGFβ suppresses the DNA synthesis of Schwann cells triggered by axons (Guenard et al., 1995). More recently IGF has also been shown to be essential for the mitogenic activity of growth factors apart from NDF in Schwann cells (Stewart et al., 1996). It should be noted that most mitogenic assays are based on the use of 2-5 day old rat Schwann cells. It is therefore not known whether, or how, the mitogenic potential of growth factors depends on the developmental age of the cells. Clarification of this issue may help to further understand the molecular interaction between Schwann cells and axons in terms of proliferation throughout peripheral nerve development.

The work described in this chapter shows that Schwann cell precursors and Schwann cells have different mitogenic responses to NDF, FGF, PDGF, TGFβ and forskolin at different developmental stages. NDF acts as a mitogen throughout both Schwann cell precursor and Schwann cell development. FGF is not a mitogen for the rat Schwann cell precursor but strongly induces DNA synthesis in Schwann cells in the presence of forskolin. Interestingly, PDGF shows no mitogenic potential for both Schwann cell precursors and Schwann cells. TGFβ can both positively and negatively control DNA synthesis in the Schwann cell precursors and Schwann cells indicating that this factor may act as regulator in nerve development. In addition, this study also demonstrates that two different mitogenic assays, in which cells are either freshly dissociated from nerves or purified in serum for 5 days, may reflect two different proliferation events during peripheral nerve development and Wallerian degeneration. Furthermore, adult Schwann cells cultured for 5 days in serum showed a strong mitogenic response to these growth factors, suggesting that intrinsic alteration in the response of adult Schwann cells to mitogens may also greatly contribute to significant proliferation of these cells during Wallerian degeneration.

Results

A transient mitogenic response to FGF-2 plus forskolin appears as precursors convert to Schwann cells.

FGF-2 in the presence of forskolin was shown not to stimulate Schwann cell precursor DNA synthesis (Chapter 3), whereas this combination promotes proliferation of Schwann cells from neonatal rats (Davis, 1992; Stewart et al., 1992). In order to know when the cells in Schwann cell lineage start to respond to this combination of mitogens, cells dissociated from E14, E15, E16, E17, E18, newborn and adult nerves were plated onto coverslips immediately after dissociation and cultured in defined medium containing FGF-2 (180pM), forskolin (5μM) and IGF-1 (13nM) for 20 hr. BrdU (20μM) was added three times during the 20 hr culture for 1.5 hr each time, and cell cultures were terminated at 5 hr, 15 hr and 20 hr respectively (as shown in Chapter 3). Cells were then double immuno-labelled with L1 and BrdU (Figure 5.1). The results showed that at the 5 hr point the DNA synthesis of E17 and E18 Schwann cells is considerably higher than that of E14 and E15 precursors and newborn cells. This result is in close agreement with the relative rates of DNA synthesis of these cells measured in vivo (Stewart et al., 1993). During the 20 hr period, DNA synthesis in E14 and E15 precursors decreased significantly, and at the 20 hr point was 0.97% and 4.66%, respectively. The same result was obtained when the FGF-2 concentration was increased from 180pM to 600 pM which is known to give maximal DNA synthesis in neonatal Schwann cells. In contrast, DNA synthesis was maintained over a 20 hr period in E17 cells and elevated in E18 cells. As expected, this combination stimulated DNA synthesis in newborn Schwann cells. Interestingly, freshly dissociated and plated adult Schwann cells from both sciatic nerve and sympathetic trunk showed no response to this combination of mitogens in this assay (Figure 5.2).

The observations so far indicated that the mitogenic activity of FGF-2 plus forskolin appeared between E15-E17, which has been defined as the period of Schwann cell

precursor to Schwann cell conversion (Jessen et al., 1994). Therefore it is most likely that FGF-2 in the presence of forskolin is a mitogen for Schwann cells but not for Schwann cell precursors. The mitogenic potential of this combination rapidly increased in E18 cells, and markedly decreased in newborn cells, and was lost in adult Schwann cells. On other hand, the mitogenic response of these cells at the 5 hr point increases from 10-15% at E14 and E15 to over 20% at E17 and E18 but falls below 10% in newborn cells and reaches zero in adult cells.

NDF retains its mitogenic activity throughout Schwann cell development

Previously, NDF has been shown to stimulate DNA synthesis of Schwann cell precursors. To understand its mitogenic potential during development, cells from E14, E15, E16, E17, E18, newborn and adult nerves were cultured in defined medium containing NDFβ-2 (120pM) and IGF-1 (13nM) for 20 hr. The control experiment was set up in defined medium containing IGF-1(13nM) only. In the case of NDFβ-2, BrdU was introduced into the cultures at the 18.5 hr point for 1.5 hr while in the case of IGF alone, BrdU was introduced in two 1.5 hr pulses at 3.5 hr and 18.5 hr (Figure 5.3). It was found that NDF consistently promoted DNA synthesis of cells from all embryonic stages but failed to stimulate DNA synthesis in adult Schwann cells from both sciatic nerve and sympathetic trunk (Figure 5.4A). Interestingly the mitogenic response curve at different developmental stages in this study exactly matched the curve obtained from experiments in vivo in which similar BrdU pulses were used both for Schwann cell precursors and Schwann cells (Stewart et al., 1993). In contrast, FGF-2 appears to be non-mitogenic for precursors and to become a mitogen during later developmental stages (Figure 5.4B); see also (Figure 5.1). In the control experiment, the rate of DNA synthesis declined rapidly during 20 hr in defined medium (Figure 5.4C).

PDGF-BB in the presence of forskolin failed to stimulate DNA synthesis in freshly dissociated Schwann cells

PDGF-BB in the presence of forskolin is a well known mitogen for Schwann cells which have been cultured in serum for several days (Eccleston et al., 1989; Stewart et al., 1991). In the present study, Schwann cell precursors and Schwann cells from E14, E18, newborn and adult nerves were exposed to PDGF-BB (5-50ng/ml) plus forskolin (5μM) immediately after dissociation from the nerve, and the 1.5 hr BrdU pulse was applied between 18.5 and 20 hr to the culture. For E14 Schwann cell precursors, PDGF-BB plus forskolin was applied on top of FGF-2 (3ng/ml) plus IGF since PDGF-BB plus forskolin was unable to support precursor survival (Figure 5.5). The results showed that the combination of PDGF-BB plus forskolin was unable to stimulate DNA synthesis either in Schwann cell precursors or Schwann cells at any developmental stage. Thus, PDGF-BB in the presence of forskolin appears to have no mitogenic effect on these cells in this type of assay.

Following serum exposure for 5 days the mitogenic activity of FGF-2 plus forskolin is more potent in adult Schwann cells than in neonatal cells

The experiments above examined the mitogenic activity of FGF, NDF and PDGF in cells which were plated on coverslips immediately after dissociation without any exposure to serum and without a significant time delay between removal from nerves and the mitogenic assay. In order to compare these results with the mitogenic response in Schwann cells from different postnatal ages under the conditions most frequently used in mitogenic assays, a series of experiments were carried out on cells that were first purified in medium containing 10% serum plus cytosine arabinoside for 4 days (see Methods). The purification procedure applied here is a modification of the previous method used by Brockes (1978), and is routinely used to obtain purified Schwann cells for proliferation and differentiation assays. This method is based on two considerations. Firstly, the presence of myelin from 4 day to adult rat Schwann cells could affect the mitogenic assay because myelin debris in culture has been shown to stimulate Schwann cell proliferation (Baichwal and DeVris, 1989; DeVris and Baichwal, 1991). Secondly, the number of fibroblasts in nerves increases dramatically after birth which could result in significant fibroblast contamination in

Schwann cell cultures (Scarpini et al., 1988). After purification, the purity of Schwann cells in cultures from newborn, 4 days and adult rats was assayed by using p75NGF-R as a Schwann cell marker, and found to be 99.87%±0.09, 99.16%±0.13 and 92.68%±0.94 respectively, The percentage of Po positive cells was reduced from 17.42%±1.1 (24 hr) to 2.04%±0.27 (5 days) after purification of adult Schwann cells, and was less than 1% in purified newborn and 4 day Schwann cells.

To compare the mitogenic potential of FGF-2 in the presence of forskolin in cells from different postnatal ages, purified Schwann cells from newborn, 4 day and adult sciatic nerves were exposed to FGF-2 plus forskolin for a total of 44 hr. BrdU was added after 24 hr for a 20 hr pulse. p75 NGF-R and BrdU double labelling was used to determine Schwann cell DNA synthesis. FGF-2 dose-response curves were constructed in a constant amount of forskolin (2μM). The experiments revealed that the mitogenic response was strikingly age-dependent (Figure 5.6A). The DNA synthesis in Schwann cells from 4 day old animals and adult animals was up to four and six fold greater, respectively, than that of cells from 1 day old animals. The peak concentration of FGF-2 was between 60-600pM. The forskolin dose-response curves were also established in constant FGF-2 (400pM). A similar age-dependent mitogenic response pattern was obtained (Figure 5.6B). These results indicate that in serum-purified cells the mitogenic potential of the combination of FGF-2 plus forskolin increases with the postnatal age of the cells (Figure 5.7).

To test whether myelin debris was likely to contribute to these unexpected differences, Schwann cells from the largely unmyelinated sympathetic trunk of adult rats were exposed to various concentrations of FGF-2 in constant forskolin. The dose- response curve obtained with these cells was similar to that of cells from the myelinated sciatic nerve of adult rats (Figure 5.8). Moreover, Po immunolabelling showed that after a total of 5 days in culture essentially no Po positive cells were seen in these cells from adult sympathetic truck and 4 day old sciatic nerves, and the percentage of Po positive cells from adult sciatic nerves in purified cultures was 2.04%±0.27 as mentioned above. These observations strongly suggest that the age-

dependent changes in mitogenic responsiveness are due to an intrinsic mechanism rather than the effect of any exogenous myelin-related substances.

Either FGF-2 or forskolin alone stimulates DNA synthesis in adult Schwann cells, but not in neonatal cells

The experiment described in the previous section indicated that the combination of FGF-2 and forskolin had a particularly strong mitogenic effect on adult Schwann cells. Therefore, it was of interest to test whether adult cells might respond to FGF-2 or forskolin alone although FGF-2 normally requires a cAMP elevating agent for stimulation of DNA synthesis. The Schwann cells were exposed to FGF-2 (0-600pM) or forskolin (0-10μM) alone for 44 hr. The BrdU was applied and the cells were labelled as described above (Figure 5.9). Surprisingly, either FGF-2 or forskolin alone stimulated DNA synthesis in these cells in a dose-dependent manner, resulting in a maximum of over 10% DNA synthesis in these cells in either forskolin (10μM) or FGF-2 (600pM). No BrdU labelling (0.2%) was detected in p75 NGF-R positive Schwann cells from adult sciatic nerves in defined medium without FGF-2 or forskolin. Furthermore, high concentrations of forskolin (10μM) or FGF-2 (600pM) also stimulated DNA synthesis in adult Schwann cells from sympathetic trunk (data not shown). In contrast to these observations, no significant DNA synthesis was observed in Schwann cells from 4 day or newborn animals when these cells were exposed to forskolin or FGF-2 alone (Figure 5.9), which confirmed a previous report that neither forskolin nor FGF-2 alone stimulated DNA synthesis in neonatal Schwann cells in defined medium (Stewart et al., 1992).

PDGF-BB combined with forskolin also shows a distinctly age-dependent mitogenic activity in Schwann cells pre-exposed to serum.

The combination of PDGF-BB and forskolin is known to be a mitogen for neonatal Schwann cells which have been cultured in serum for more than 5 days (Eccleston et al., 1990; Davis., 1990; Stewart et al., 1992). In freshly dissociated Schwann cells, however, this combination failed to stimulate DNA synthesis in precursors or in

Schwann cells from E18, newborn and adult nerves as shown above. To test whether serum exposure could also affect the mitogenic responses of this combination, dose-response curves were constructed for PDGF-BB in the presence of a constant forskolin levels (2μM) and for forskolin in the presence of constant PDGF-BB(5ng/ml) in Schwann cells purified in serum for 5 days (Figure 5.10). The results showed that Schwann cells do respond to this combination after exposure to serum. Strikingly, the responses of adult and 4 day Schwann cells were up to 10 and 5 fold higher, respectively, than that of newborn cells. In general, the mitogenic activity of PDGF-BB in the presence of forskolin was smaller than that of FGF-2 in the presence of forskolin.

Mitogenic activity of TGFβ in the Schwann cell lineage

Previously, TGFβ has been shown to act as a mitogen for Schwann cell precursors in the FIF medium (FGF-2 180pM plus IGF-1 13nM and forskolin 5μM) (Chapter 3). To understand the mitogenic response to TGFβ during development, cells from E14, E18 and newborn sciatic nerves were plated on coverslips immediately after dissociation as previously described for the culture of embryonic cells. The cells were exposed to both FIF medium and FIF medium plus TGFβ1 (1ng/ml)_for 20 hr with a 1.5 hr BrdU pulse at 18.5 hr. In the case of precursors, it was found that when TGFβ was added to FIF medium TGFβ acted as mitogen even though FGF-2 plus IGF and forskolin did not stimulate DNA synthesis in these cells as shown previously (Figure 5.11). However, TGFβ slightly suppressed the mitogenic activity of FGF-2 plus IGF and forskolin in Schwann cells from E18 and newborn rats (Figure 5.12A). These experiments suggested that TGFβ may act as both positive and negative regulator to control cell proliferation depending on the proliferative situation of these cells. To further test this idea, TGFβ was used on top of NDFβ-2 (400pM) plus IGF-1 (13nM) in the Schwann cell precursor DNA synthesis assay. In one experiment, TGFβ1 (1ng/ml) significantly suppressed DNA synthesis triggered by NDFβ plus IGF (Figure 5.13, 5.14).

To explore the mitogenic potential of TGFβ in Schwann cells from postnatal stages, Schwann cells from 1 day, 4 day and adult rat nerves were purified for 5 days in serum containing medium as described above. The cells were then exposed to FGF-2 (600pM) plus forskolin (2μM) or this combination plus TGFβ1 (1ng/ml) for 44 hr in total. BrdU was introduced into the culture for the last 20 hr (Figure 5.12B). The results showed that in these culture conditions, the addition of TGFβ1 caused a 2.5 fold increase in DNA synthesis in newborn Schwann cells, in contrast to previous findings in which Schwann cells were freshly dissociated from nerves. Slight suppression of DNA synthesis triggered by FGF-2 plus forskolin was seen in adult cells, while the effect of TGFβ on top of FGF-2 plus forskolin in 4 day Schwann cells was insignificant.

Pre-exposure to serum affects the mitogenic response of Schwann cells to growth factors

One of the more striking observations described in the previous sections concerns the mitogenic responses of adult Schwann cells. They did not respond to any previously known mitogens in the assay performed on freshly dissociated cells, while they showed a vigorous mitogenic response to the same mitogens after being cultured in serum for 5 days, although the BrdU assay applied in these two experiments was different. It is possible that the growth factors present in serum may alter Schwann cell properties. To test this possibility directly, Schwann cells freshly dissociated from adult and newborn rats were cultured in FGF-2 (180pM) plus forskolin (5μM) for 2 days, while in another experiment, Schwann cells from adult and newborn were cultured in serum for 5 days then exposed to FGF-2 (180pM) plus forskolin (5μM) for a further 2 days. The cells from both experiments were dosed with BrdU for the last 20 hr of the 2 or 7 day assays (Figure 5.15). Using this comparable BrdU assay, the results showed that in cells from newborn rats the DNA synthesis in response to FGF-2 plus forskolin fell from 51.26%±0.55 to 21.22%±2.88 after treatment with serum. Interestingly, in Schwann cells from adult rat, the DNA synthesis response increased from 6.19%±1.99 to 73.13%±3.68 after the same treatment in serum

(Figure 5.15). Therefore, it is clear that pre-exposure to serum can radically alter the mitogenic response of Schwann cells.

The length of the culture period determines the mitogenic response

During the purification procedure Schwann cells were not only exposed to serum but also cultured for 5 days. It was of interest to know whether the length of the culture period alone would also affect the mitogenic response even in the absence of serum. Cells dissociated from newborn and adult rat nerves were firstly cultured in serum free defined medium for 0, 1, 2, 3, 4 and 5 days respectively. FGF-2 (180pM) plus forskolin (5μM) was applied to each group. The BrdU incorporation was monitored in the last 20 hr of a total 2 day incubation period of FGF plus forskolin as previously described (Figure 5.16). The results showed that the mitogenic response to this combination diminished rapidly with time in newborn cells. In contrast, the mitogenic response increased gradually in adult cells. It is instructive to compare these results with those obtained when newborn and adult cells were exposed to serum for 5 days prior to a 2 day exposure to FGF plus forskolin (see Figure 5.15). The comparison shows that the 5 day period has similar qualitative effects irrespective of whether serum is present or not i.e. the mitogenic responsiveness of newborn cells decreases and that of adult cells increases. The magnitude of these changes, however, is much greater in the presence of serum.

Discussion

It is important to define the molecular regulation of Schwann cell proliferation during development and Wallerian degeneration. The present study clearly shows that the mitogenic potential of FGF, NDF, PDGF and TGFβ differs in Schwann cell precursors and Schwann cells at different developmental stages, indicating that the role that a given growth factor plays in Schwann cell proliferation may vary during development. The differences in mitogenic responses between cells assayed soon after plating and cells assayed following 5 day exposure to defined medium or serum suggest that the culture period and culture conditions might alter the intrinsic biological properties of Schwann cells. The two different mitogenic assays used in this study (on freshly plated cells or on serum-exposed cells) provide two different ways of understanding the proliferative behaviour of Schwann cells during normal development and Wallerian degeneration.

The alteration of biological activity of Schwann cells in culture

Purification of Schwann cells in culture was established in the middle of the 1970s. There are two main Schwann cell purification methods which have been used in recent studies. In one method DRG explants were cultured for about a month, then Schwann cells were harvested by excision of the center of the explant (Wood and Bunge., 1976). The other method involved Schwann cells being directly dissociated from nerves and cultured in serum for 4-5 days (Brockes et al., 1979). Both of these methods involve culturing Schwann cells for a period and exposing them to serum. It has been unknown whether these culture conditions affect the biological activity of Schwann cells although it is clear that Schwann cells generated by these methods seem to respond to mitogens and can also be induced to myelinate (Raff. M., 1978; Salzer et al., 1980; Mirsky et al., 1980; Sobue and Pleasure., 1984; Ratner et al., 1985; 1986; Eccleston et al., 1989; Stewart et al., 1990; Morgan et al., 1991).

The experiments presented here were based on two different methods. Firstly, Schwann cell precursors or Schwann cells were immediately exposed to mitogens after dissociation from nerves without any purification. Most experiments of this type were carried out on cells from embryonic stages, at which no myelin and few fibroblasts are present in peripheral nerves (Scarpini et al., 1988; Mirsky and Jessen, 1990; Jessen and Mirsky, 1991). The purity of the cells in such cultures is 95%±1.3 at E14 and 93%±1.4 at E18. To monitor the DNA synthesis during a 20 hr assay. BrdU (1.5 hr pulse) was added three times into the culture or in some experiments during the last 1.5 hr period, between 18.5 to 20 hr only. This assay showed clearly that DNA synthesis of these cells was elevated or remained at the same levels if a mitogen was applied. In contrast, the rate of DNA synthesis rapidly declined when non-mitogens were used. The percentage of DNA synthesis of these cells based on this type of assay was about 13% in E14 precursors, 35% in E 18 Schwann cells , 10% in newborn cells and 0.2% in adult cells in response to NDF or FGF plus forskolin at the 20 hr point. The magnitude of the mitogenic response at different developmental stages correlated well with the amount of DNA synthesis seen in vivo when a similar BrdU pulse (2.4 hr) was applied to living animals (Stewart et al., 1993). It is likely that this type of assay has the following characteristics. Firstly the cells synthesize DNA during the first 1.5 hr BrdU pulse primarily in response to the mitogens they were exposed to in vivo prior to dissociation; secondly, the cells synthesize DNA during the second and particularly the third pulse primarily in response to mitogenic input in the culture dish; and thirdly, this mitogenic response to applied growth factor is similar to the response that the cells would show in vivo, while the response of the cells that have been exposed to serum for 4-5 days (see below) are likely to be affected by some factors in serum and by time in vitro. Therefore, growth factors were tested in this assay in the hope of understanding the mitogenic potential of these factors in normal peripheral nerve development.

Secondly, Schwann cells were dissociated and cultured in serum medium for 5 days, giving rise to pure cultured Schwann cells. Schwann cells from adults generated by this method showed a great increase in responsiveness to mitogens such as NDF, FGF, PDGF-BB and forskolin (from 6% to 73%), whereas a significant reduction in

responsiveness to the same mitogens was seen in Schwann cells from newborn animals (from 51% to 25%). This response to mitogens poorly reflects the proliferation tendency of these Schwann cells in normal nerves, since it is well known that adult Schwann cells are quiescent in normal nerves. On the other hand, the change in mitogenic response during culture matches the alteration of these cells in terms of mitogenic response during Wallerian degeneration both of newborn and adult nerves. Previous experiments indicated that transecting neonatal sciatic nerves resulted in a decrease in DNA synthesis in these Schwann cells (Komiyama and Suzuki, 1992). In contrast, transecting adult sciatic nerves led to massive proliferation of adult Schwann cells (Clemence et al., 1989; Komiyama and Suzuki, 1992). It was suggested that myelin debris and macrophage participation might be the cause of this. Nevertheless, the observations in the present study indicate that Schwann cells cultured in serum or defined medium for several days change their mitogenic response, closely mimicking the response of Schwann cells during Wallerian degeneration. Although it is still unclear what causes this alteration in terms of molecular regulation in these cells, one possible explanation is that lack of axonal signals in these cultures may result in changes in the expression of growth factor receptors.

The present study not only shows that Schwann cells in culture change their mitogenic response but also indicates that the length of the culture period determines this alteration since Schwann cells cultured in serum or defined medium changed their responsiveness in a similar direction, although the presence of serum in the culture medium significantly affected the extent of these changes. In conclusion, it is most likely that a study of Schwann cells which have been cultured for several days in the absence of neuronal signals is more conducive to understanding the biological activity of Schwann cells during Wallerian degeneration, whereas a study of Schwann cells which are tested immediately after dissociation from nerves leads to an understanding of the biological activity of Schwann cells in normal nerves.

Mitogenic responses in the Schwann cell lineage

Many growth factors have been found to stimulate DNA synthesis in Schwann cells in vitro (Davies, 1991; Stewart et al., 1992). It is noteworthy that most mitogenic assays have been carried out on neonatal Schwann cells which were cultured in serum containing medium for several days before the start of the assay. As discussed above, it is unclear how these results can be directly related to factors in the nerve that might promote Schwann cell proliferation throughout development.

Mitogenic responses to growth factors during development

The present experiment in which Schwann cells were immediately exposed to growth factors after fresh dissociation from the nerve showed that NDF, FGF, PDGF and TGFβ act differently on Schwann cells in terms of mitogenic activity at different developmental stages. FGF in the presence of forskolin is non-mitogenic for Schwann cell precursors, and then gradually becomes a mitogen for Schwann cells. The mitogenic potential of this combination reaches a peak at E18, dropping significantly by the newborn stage. In this assay, adult Schwann cells do not respond to this combination. In contrast, TGFβ on top of FGF and forskolin stimulates DNA synthesis in Schwann cell precursors, and then its mitogenic potential is gradually lost when it is added on top of FGF and forskolin. As FGF plus forskolin becomes increasingly mitogenic for Schwann cells TGFβ rather than stimulating DNA synthesis, slightly inhibits mitogenic activity triggered by FGF plus forskolin. In adult cells, TGFβ did not stimulate DNA synthesis in any of the growth factor combinations tested. NDF seems to be a consistent mitogen throughout early Schwann cell development, its activity being gradually reduced in postnatal cells. In adult cells, NDF had no mitogenic potential. PDGF-BB in the presence of forskolin, a typical mitogen for longer term cultured Schwann cells (Eccleston et al., 1992; Stewart et al., 1992), was unable to stimulate DNA synthesis in this assay, suggesting that this combination may not contribute to proliferation of Schwann cells in normal nerves. It was a consistent finding that the mitogenic potential of all of these growth factors was lost in adult Schwann cells although they showed varying mitogenic effects on cells from more immature animals. Some evidence indicates that Schwann cells in the adult nerve express low levels of PDGF receptor, FGF receptor and ErbB2

mRNA or protein (Eccleston et al., 1992; Reddy and Pleasure, 1992; Cohen et al., 1992; Jin et al., 1993; Davis, 1993). Therefore, one possibility is that down-regulation of corresponding receptors in the adult may lead to the loss of response in these cells to growth factors.

Mitogenic responses to growth factor during Wallerian degeneration

When experiments were done in a different way by using Schwann cells mainly from postnatal stages cultured in serum for 5 days, different results were obtained. Under these conditions, the mitogenic potential of FGF plus forskolin, was much greater in adult Schwann cells than in younger cells. Moreover, it was found that FGF was sufficient on its own to stimulate DNA synthesis in adult Schwann cells. PDGF-BB plus forskolin, as expected, was mitogenic for these Schwann cells and showed an age-dependent mitogenic response. It seems likely that the mitogenic response of adult Schwann cells to all growth factors is upregulated under these culture conditions. It is possible that this can be related to the vigorous division of these cells seen during Wallerian degeneration.

Effects of TGFβ on cells in Schwann cell lineage

TGFβ, as mentioned above, slightly suppressed DNA synthesis triggered by FGF plus forskolin in freshly dissociated newborn Schwann cells. In contrast, it promoted the mitogenic potential of FGF plus forskolin in the same newborn Schwann cells following cultured in serum containing medium for 5 days. One of the differences between freshly dissociated cells and serum-exposed cells is that the mitogenic response to FGF plus forskolin was reduced (from 51% in freshly dissociated cells to 25% in serum-exposed cells). In addition, TGFβ suppressed the high DNA synthesis generated by FGF plus forskolin in serum-exposed adult Schwann cells. Taken together, it seems most likely that the positive or negative effect of TGFβ on Schwann cells depended on proliferation status of cells. When the cells are quiescent or the proliferative response of these cells to FGF plus forskolin is declining, TGFβ synergizes with FGF plus forskolin to stimulate DNA synthesis in these cells. When

Schwann cells were dividing at a maximum rate TGFβ lowers the proliferation rate. TGFβ also inhibited DNA synthesis in Schwann cells driven by neurons (Chandross et al., 1995). The positive and negative regulation by TGFβ was also observed in Schwann cell precursors. TGFβ promoted precursor DNA synthesis in the presence of the non-mitogenic combination of FGF-2 plus forskolin, but inhibited DNA synthesis in the mitogenic agent NDFβ. Thus, TGFβ is able to act as both a positive and negative regulator of proliferation of the cells in the Schwann cell lineage. A study on 3T3 cells indicated that TGFβ might downregulate PDGF receptors in these cells resulting in a decreased mitogenic response to PDGF-AA (Gronwald et al., 1989). Therefore, it is also possible that the positive and negative regulation by TGFβ in Schwann cells may also relate to the receptor regulation in these cells by TGFβ.

The potential for mitogenic response in adult Schwann cells

Culturing adult Schwann cells has always been thought to be difficult due to fibroblast contamination and the effects of myelin debris (Yoshino et al., 1987; Scarpini et al., 1988; Baichwal and DeVries, 1989; Morrissey et al., 1991). The adult Schwann cells generated in this study were 93% pure, confirmed by both p75 NGF-R and S-100 staining. Although this purity is slightly less than that of previous reports in which purified adult Schwann cells were obtained by processing in culture for more than 1 month (Morrissey et al., 1991) it greatly simplified the culture method for adult Schwann cells. Adult Schwann cells cultured for 5 days in serum containing medium, showed a strong response to known mitogens. It is clear that this response does not relate to the effects of myelin debris since the Po positive adult Schwann cells were less than 2% after 6 days in culture, or fibroblast contamination (only 7% in these cultures). Meanwhile non-myelinating Schwann cells from the adult sympathetic trunk showed a similar mitogenic response pattern to those derived from the sciatic nerve. These observations suggest that intrinsic changes which occur in these Schwann cells in response a denevation may lead to the massive DNA synthesis in response to growth factors and that the effect of exogenous myelin debris is secondary to the response. These intrinsic changes in Schwann cell biological activity

may contribute to the strong proliferation of adult Schwann cells seen during Wallerian degeneration.

Although the present study does not examine the changes in growth factor receptors that may occur when these cells are cultured, related studies show that adult Schwann cells in normal nerves express very low levels of ErbB2 mRNA and protein. After sciatic nerve transection Schwann cells in the distal stump expressed high levels of this protein and mRNA (Cohen et al., 1992). Therefore, it is possible that corresponding growth factor receptors such as PDGF receptor, FGF receptor and ErbB2 which are expressed in very low levels in adult Schwann cells in vivo (Eccleston et al., 1992; Reddy and Pleasure, 1992; Cohen et al., 1992; Jin et al., 1993), could be upregulated in cultures and during Wallerian degeneration. This receptor regulation may act as an intrinsic control on Schwann cell proliferation during development and Wallerian degeneration.

Recently, two separate groups have reported on the mitogenic responses of adult mouse Schwann cells. It was found that Schwann cells from adult mouse did not respond to any growth factor combinations except GGF in high concentrations when these cells were cultured in mixed serum and serum free medium for 4 days on a collagen-coated substrate (Zhang et al., 1995). It was also found that Schwann cells from adult mouse did respond to FGF alone, forskolin alone and their combination when these cells were cultured in serum medium for 3 more weeks (Watabe et al., 1994). These different mitogenic responses of adult Schwann cells may be due to the culture period and serum exposure since the present study has indicated that the culture period determines the mitogenic response of adult Schwann cells and serum exposure significantly enhances the mitogenic potential of these cells. Moreover, this time-dependent mitogenic responsiveness can also be seen in transected adult nerve, since 3 post-transectional days are necessary for the proliferation of the adult Schwann cells in the distal stump to reach a maximum level (Komyama and Suzuki, 1992). It would therefore, be of interest to know whether the culture period and serum exposure are related to receptor regulation which leads to the proliferation of Schwann cells.

Figure 5.1. Developmental regulation of the mitogenic potential of FGF-2 plus forskolin in cells of the Schwann cell lineage

Cells dissociated from E14, E15, E17, E18, newborn and adult rat sciatic nerves were immediately cultured in FIF medium [FGF-2 (180pM) plus forskolin (5μM) and IGF-1 (13nM)] for 20 hr. A 1.5 hr BrdU pulse was used at the 3.5 hr, 13.5 hr and 18.5 hr points. BrdU and L1 antibodies (or S100, p75NGF-R for postnatal stage) were used to measure the DNA synthesis in identified cells. The results show that the mitogenic response to FGF-2 plus forskolin is absent in E14 and E15 precursors and adult Schwann cells, and present in E17, E18 and NB Schwann cells.

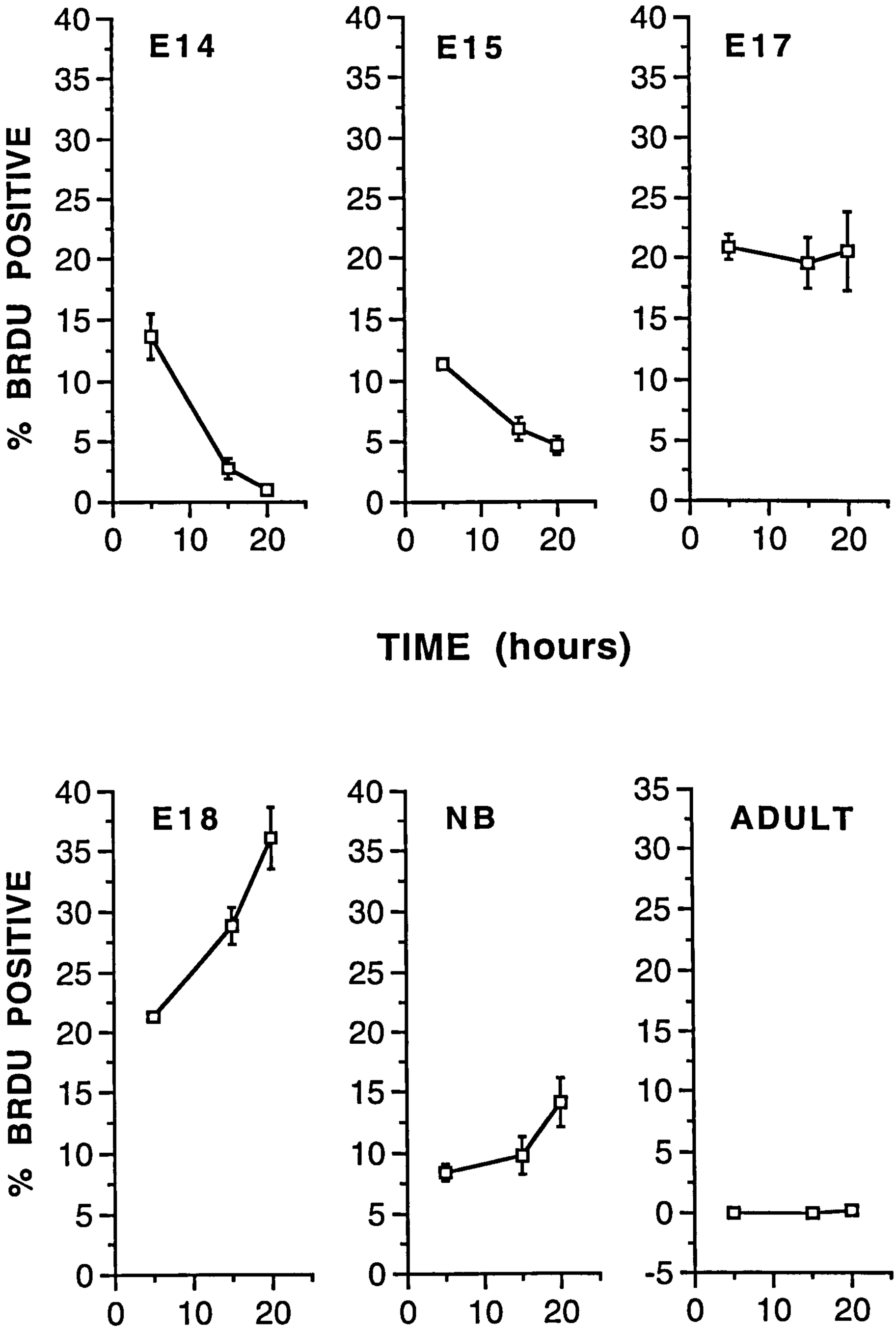

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Figure 5.2 FGF-2 in combination with forskolin stimulates DNA synthesis in E18 and NB Schwann cells, but not in E14 precursors or adult Schwann cells.

Cells were exposed to FGF-2 plus forskolin and IGF-1 immediately after dissociation from nerves for 20 hr. A 1.5 hr BrdU pulse was used at the 18.5 hr point. E14 Schwann cell precursors (A) and E18 Schwann cells (C) were labelled with L1. NB Schwann cells (E) were labelled with p75NGF-R. Adult Schwann cells (G) were labelled with S100. B,D,F,H show the BrdU labelling in corresponding to A,C,E,G. Magnification 600X

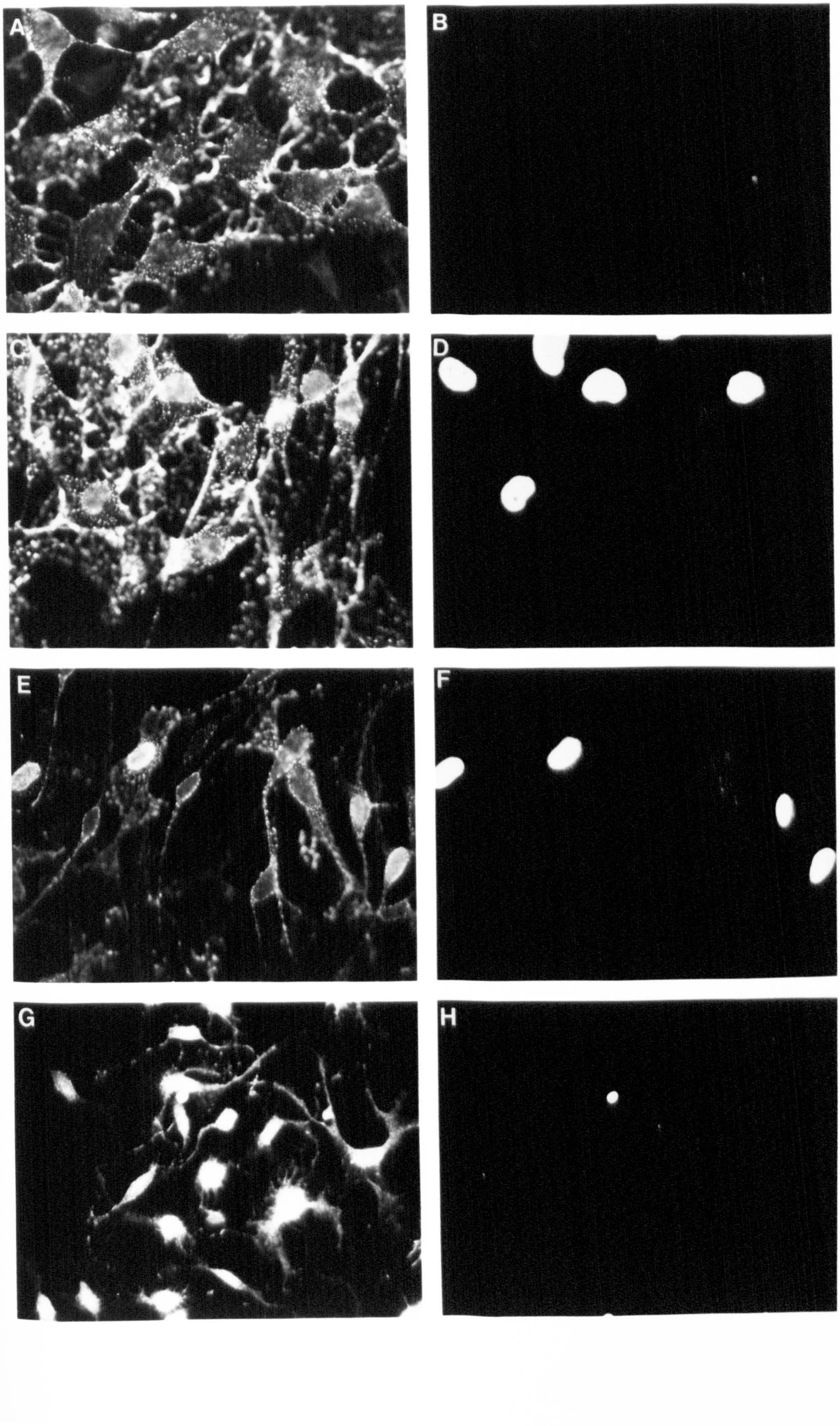
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Figure 5.3 NDFβ stimulates DNA synthesis in E14 Schwann cell precursors and E18, NB Schwann cells, but not in adult Schwann cells.

Cells were exposed to NDFβ plus IGF-1 immediately after dissociation from nerves for 20 hr. A 1.5 hr BrdU pulse was used at the 18.5 hr point. E14 Schwann cell precursors (A) and E18 Schwann cells (C) were labelled with L1. NB Schwann cells (E) were labelled with p75NGF-R. Adult Schwann cells (G) were labelled with S100. B,D,F,H were the BrdU labelling in corresponding to A,C,E,G. Magnification 600X

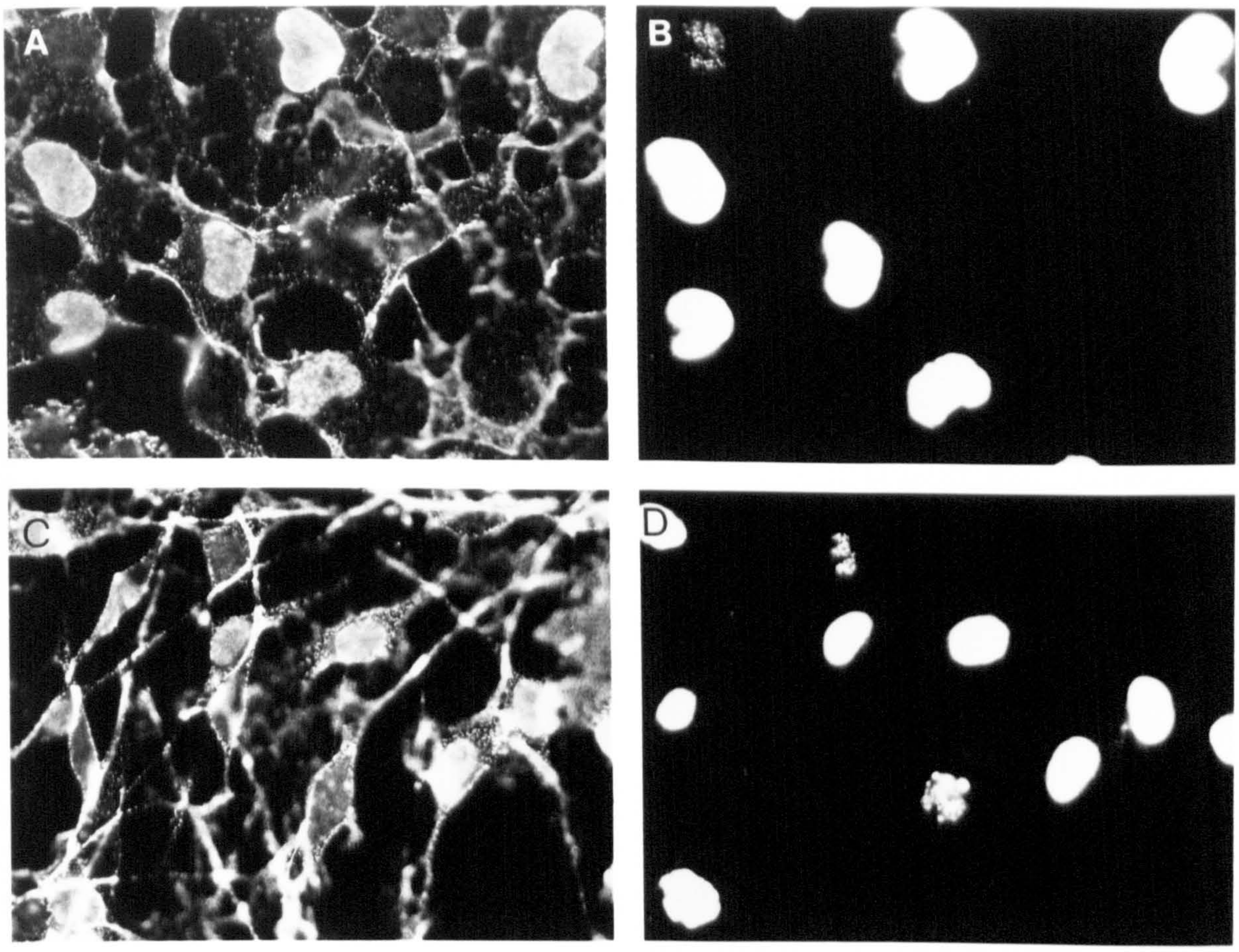

A
B
C
D
E
F
G
H

Figure 5.4 Developmental regulation of the mitogenic potential of NDFβ-2 in cells of the Schwann cell lineage

Cells dissociated from E14, E15, E16, E17, E18, newborn and adult rat sciatic nerves were immediately cultured in defined medium containing (A) NDFβ (400pM) plus IGF-1 (13nM), (B) FGF-2 (180pM) plus forskolin (5μM) and IGF-1 (13nM) taken from Figure 5.1 at 20 hr point, (C) IGF (13nM) control at 20 hr. A 1.5 hr BrdU pulse was used at the 18.5 hr point or the 3.5 hr point for IGF only. The results show that NDFβ, but not FGF-2 plus forskolin, stimulates DNA synthesis in E14 and E15 precursors. Both NDFβ and FGF-2 plus forskolin are mitogenic for embryonic and newborn Schwann cells, but have no mitogenic activity for adult Schwann cells. In IGF alone, DNA synthesis in these cells decreased dramatically from the 5 hr point to the 20 hr point at all ages from E16 to adult.

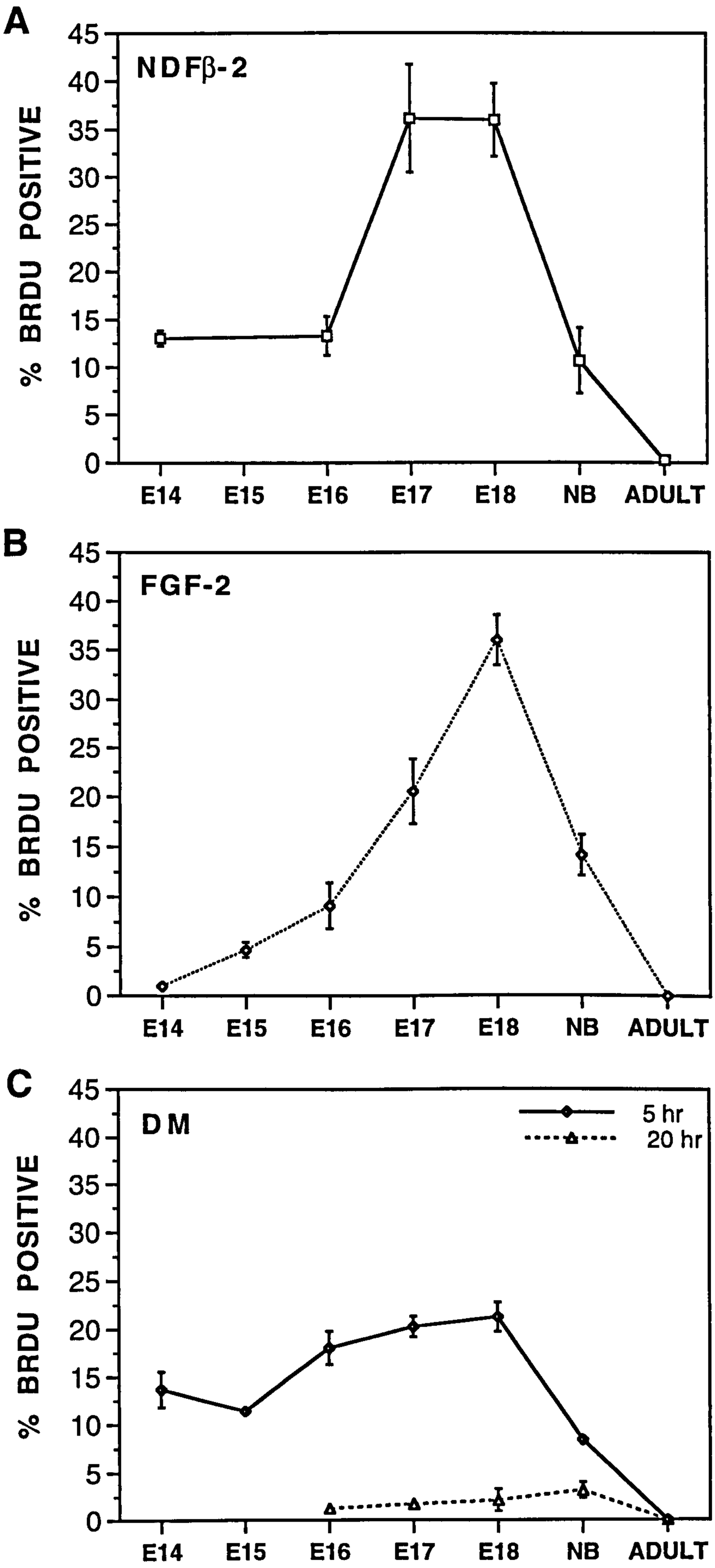

A
NDFβ-2
% BRDU POSITIVE
45
40
35
30
25
20
15
10
5
0
E14
E15
E16
E17
E18
NB
ADULT
B
FGF-2
% BRDU POSITIVE
45
40
35
30
25
20
15
10
5
0
E14
E15
E16
E17
E18
NB
ADULT
C
D M
5 hr
20 hr
% BRDU POSITIVE
45
40
35
30
25
20
15
10
5
0
E14
E15
E16
E17
E18
NB
ADULT

Figure 5.5 The mitogenic potential of PDGF-BB plus forskolin in cells of the Schwann cell lineage

Cells dissociated from E14, E18, newborn and adult rat sciatic nerves were immediately cultured in defined medium containing PDGF-BB (50ng/ml) plus forskolin (5μM) and IGF-1 (13nM) for 20 hr. In E14 precursor cultures, FGF-2 (180pM) was included since precursors die in PDGF-BB alone. A 1.5 hr BrdU pulse was used at the 18.5 hr point. The results show that DNA synthesis of these cells in PDGF-BB plus forskolin medium is similar to that seen in IGF alone medium (Figure 5.4C).

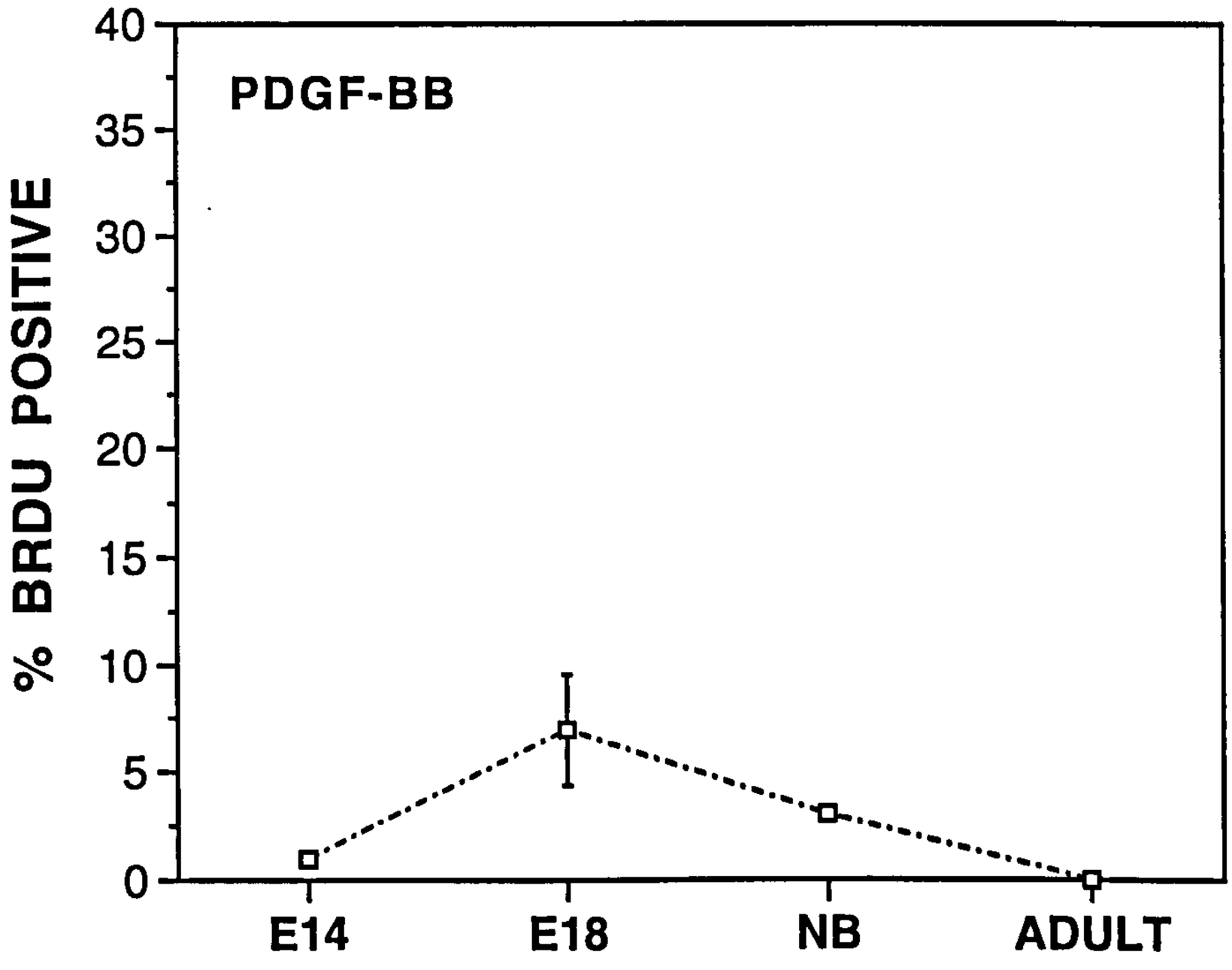
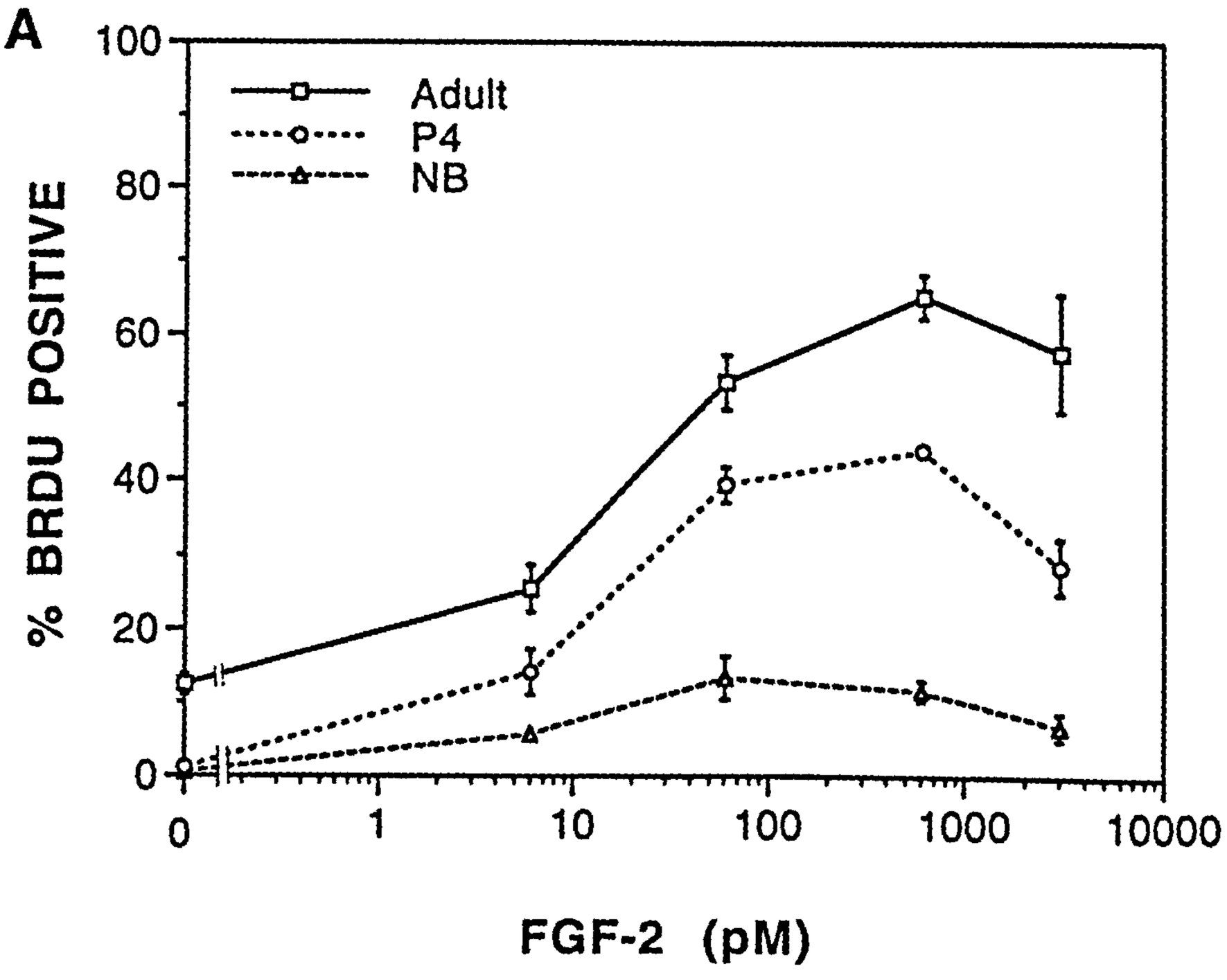

PDGF-BB
% BRDU POSITIVE
40
35
30
25
20
15
10
5
0
E14
E18
NB
ADULT

Figure 5.6 Mitogenic potential of FGF-2 plus forskolin in Schwann cells pre-cultured in serum-containing medium for 5 days.

Schwann cells from newborn, postnatal day 4 (P4) and adult sciatic nerves were cultured in serum-containing medium for 5 days, then the purified Schwann cells were exposed to (A) various concentrations of FGF-2 in constant of forskolin 2μM, (B) various concentrations of forskolin in constant FGF-2 (400pM) for a total of 44 hr. BrdU was used for the last 20 hrs of the assay. The results show that DNA synthesis stimulated by FGF-2 plus forskolin is higher in adult Schwann cells than that seen in P4 and NB Schwann cells.


A
100
80
60
40
20
0
% BRDU POSITIVE
Adult
P4
NB
0
1
10
100
1000
10000
FGF-2 (pM)

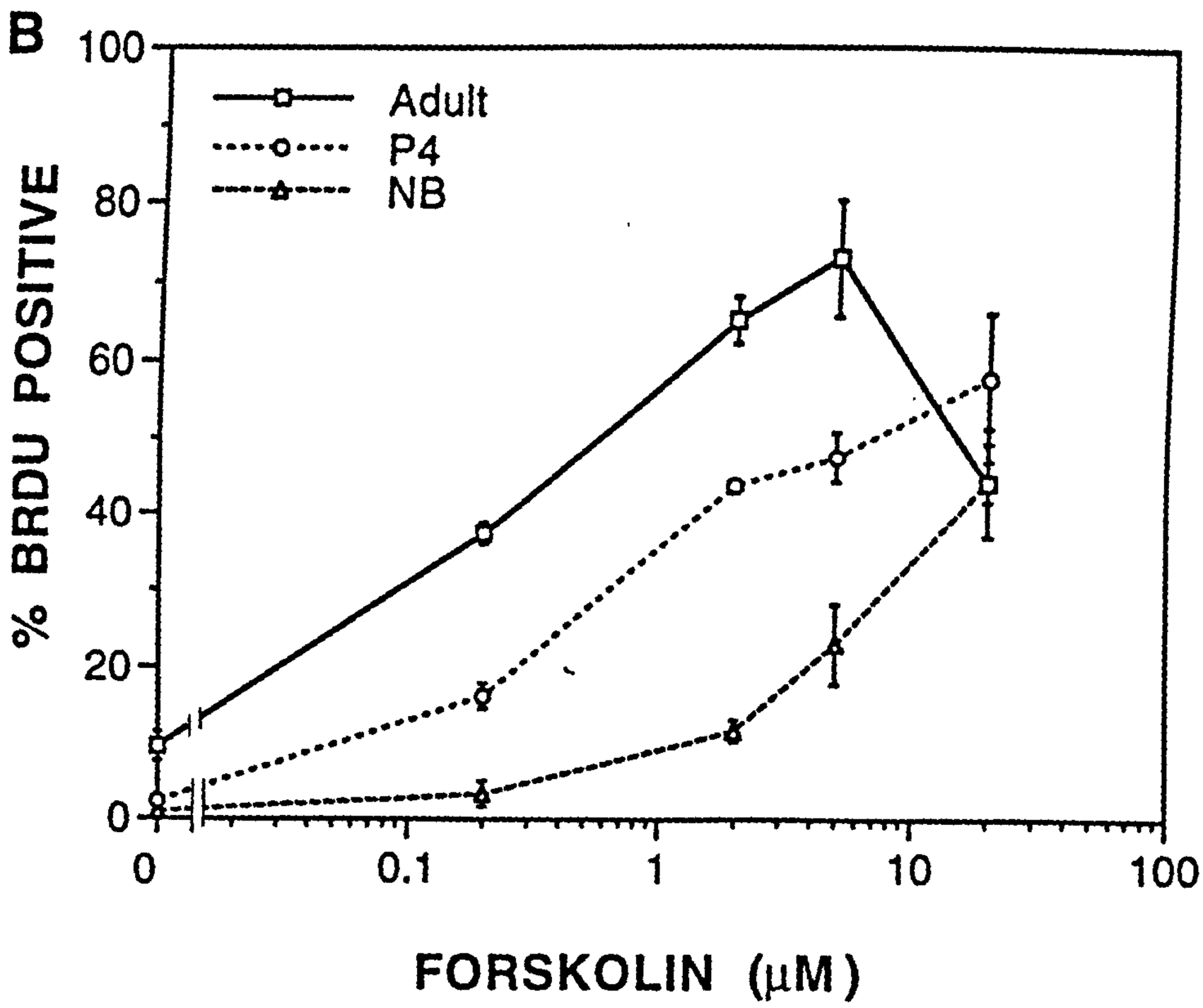

B
100
80
60
40
20
0
% BRDU POSITIVE
Adult
P4
NB
0
0.1
1
10
100
FORSKOLIN (μM)

Figure 5.7 Mitogenic activity of FGF plus forskolin in adult Schwann cells was higher than that in younger cells.

Schwann cells from adult (A,B), postnatal day 4 (C,D) and newborn (E,F) rat sciatic nerves were pre-cultured in serum-containing medium for 5 days, and then the purified cells were exposed to FGF-2 plus forskolin for 44 hr. A 20 hr BrdU pulse was used for the last 20 hr assay. Cells were double-labelled with p75NGF-R (A,C,E) and BrdU (B,D,F). Magnification 500X

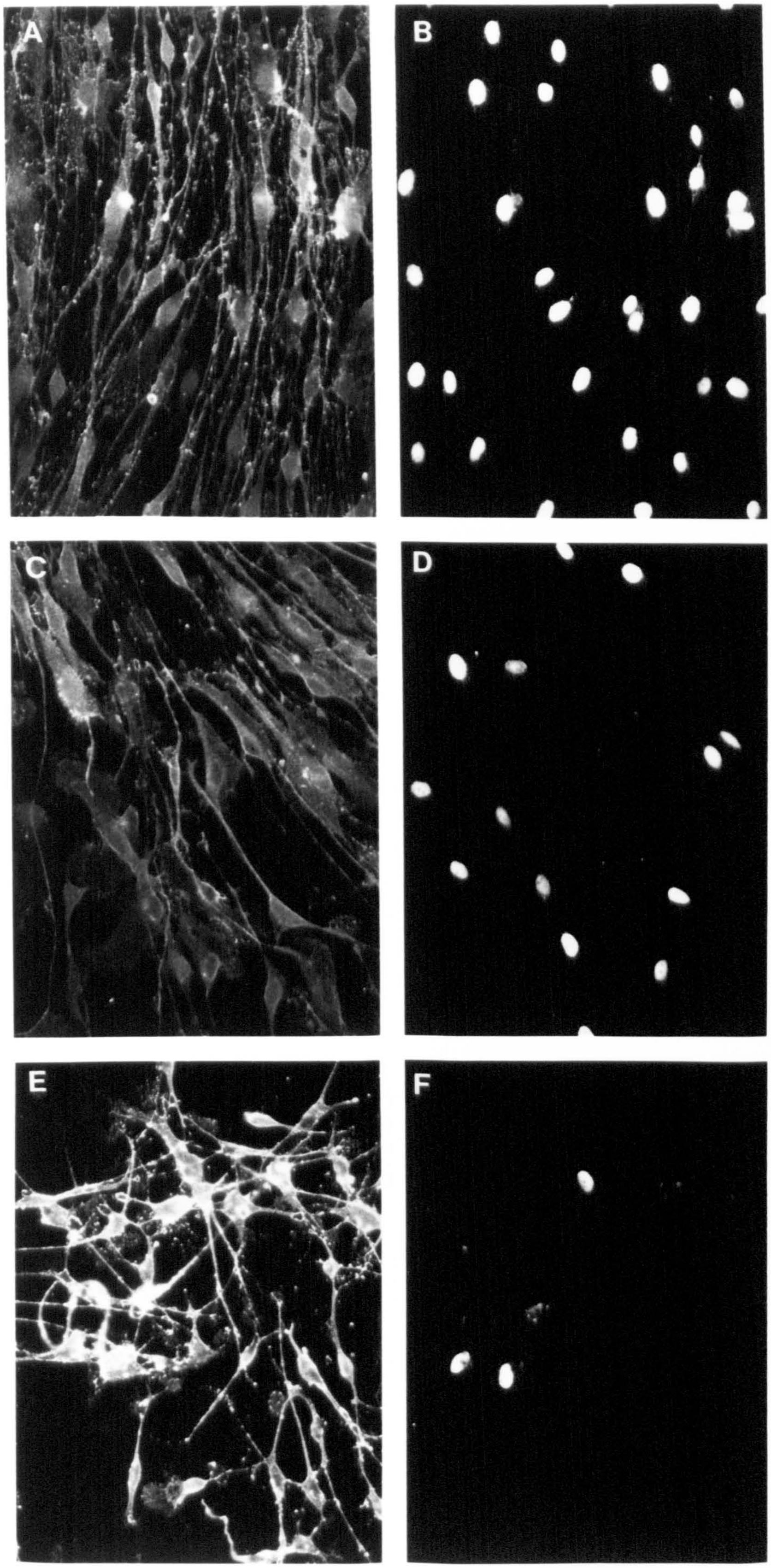

A
B
C
D
E
F

Figure 5.8 Mitogenic potential of FGF-2 plus forskolin was similar in Schwann cells from both adult sciatic nerves and sympathetic trunk nerves

Schwann cells from both adult sciatic nerves and sympathtic trunk nerves were cultured in serum-containing medium for 5 days. The purified Schwann cells were then exposed to various concentrations of FGF-2 in constant of forskolin, 2 μM for 44 hr. BrdU was used for the last 20 hr. The results show that Schwann cells from both adult sciatic nerves and sympathetic trunk nerves have a similar response to the combination of FGF-2 plus forskolin.

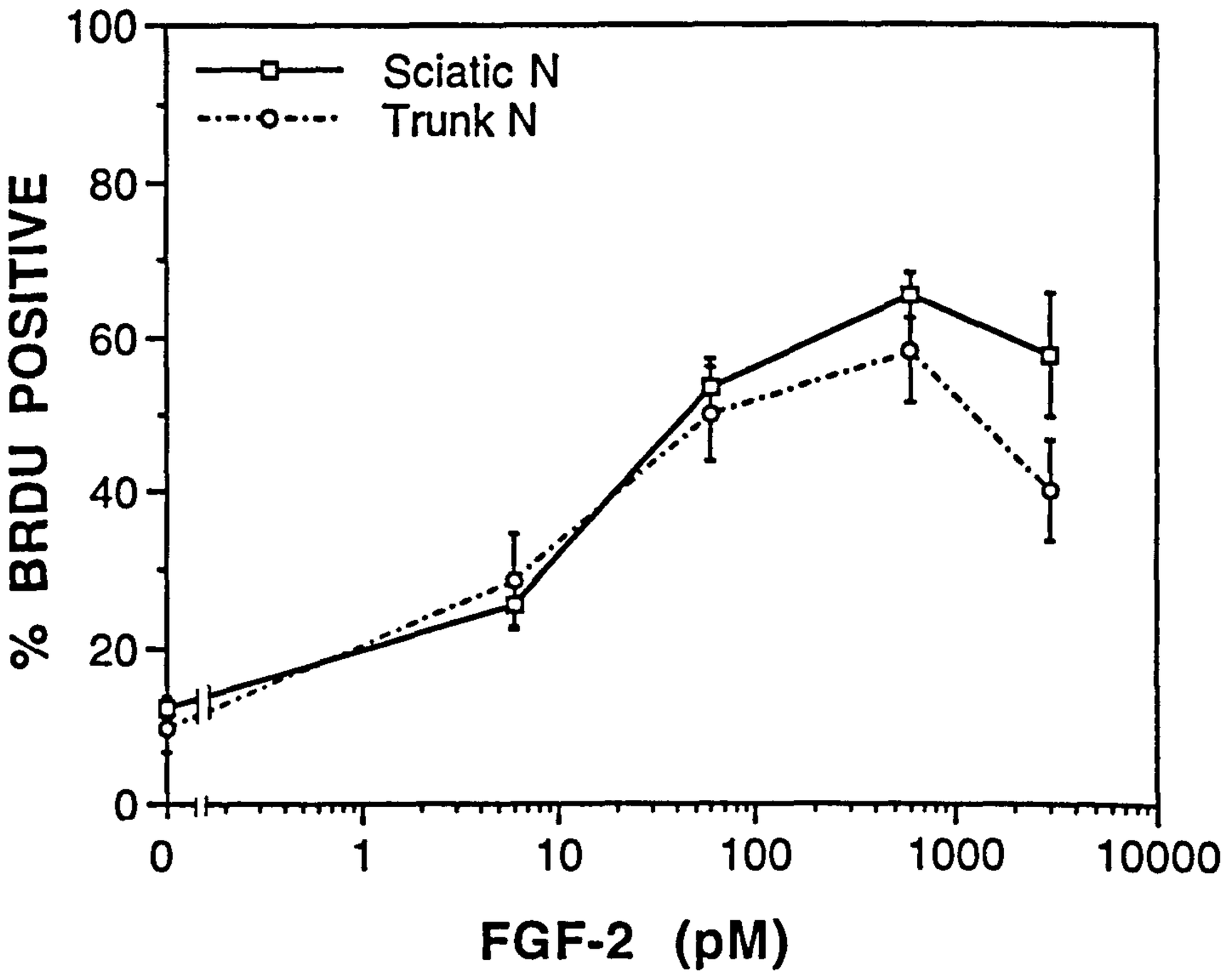
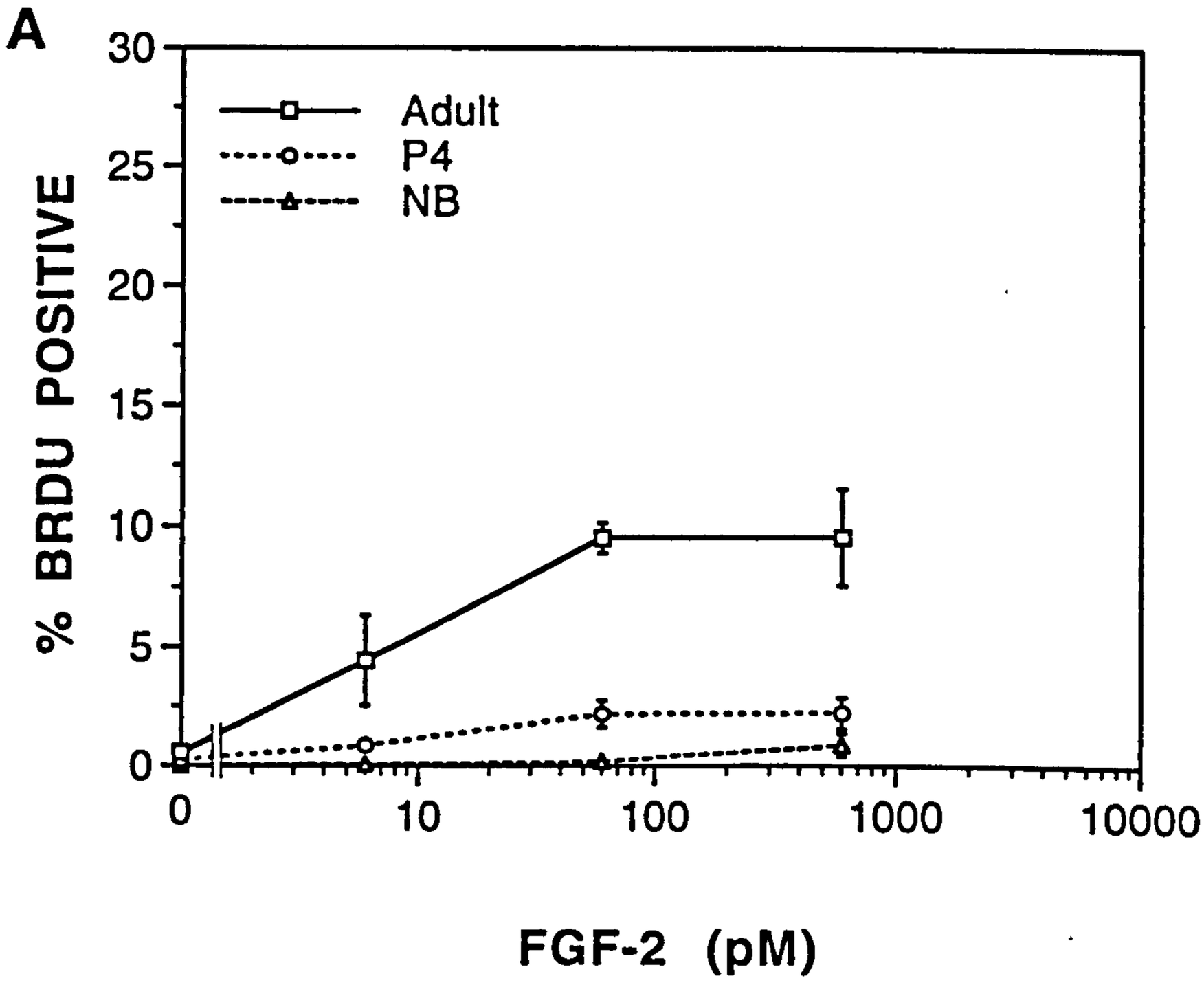

100
80
60
40
20
0
% BRDU POSITIVE
Sciatic N
Trunk N
0
1
10
100
1000
10000
FGF-2 (pM)

Figure 5.9 Mitogenic potential of FGF-2 or forskolin alone in Schwann cells pre-cultured in serum-containing medium for 5 days.

Schwann cells from newborn (NB), postnatal day 4 (P4) and adult sciatic nerves were cultured in serum-containing medium for 5 days, then the purified Schwann cells were exposed to various concentrations of FGF-2 (A) or forskolin (B) for 44 hr. BrdU was used for the last 20 hr of the assay. The results show that FGF-2 or forskolin alone acts as a dose-dependent mitogen for adult Schwann cells, but not for NB and P4 Schwann cells.


A
30
25
20
15
10
5
0
% BRDU POSITIVE
Adult
P4
NB
0
10
100
1000
10000
FGF-2 (pM)

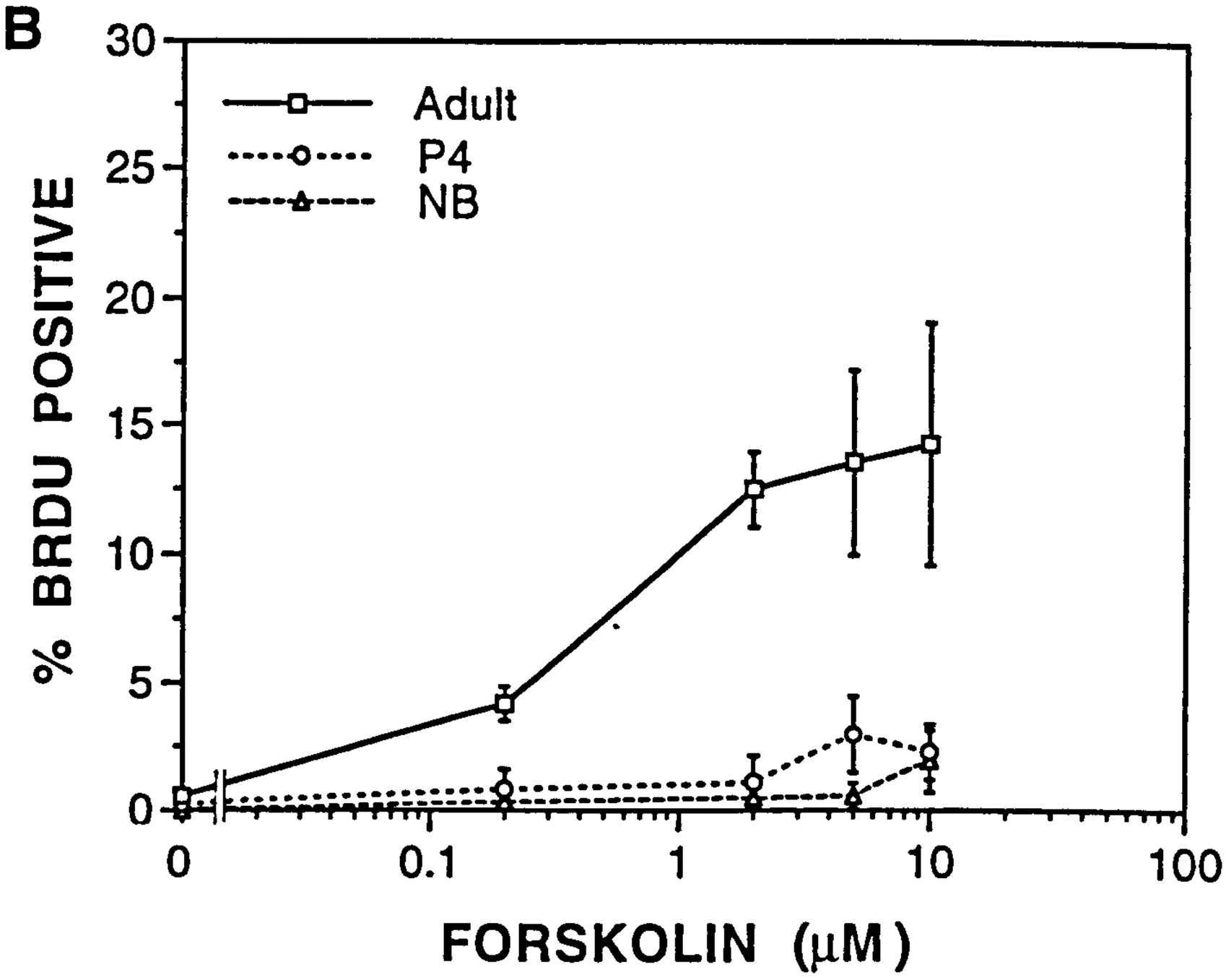
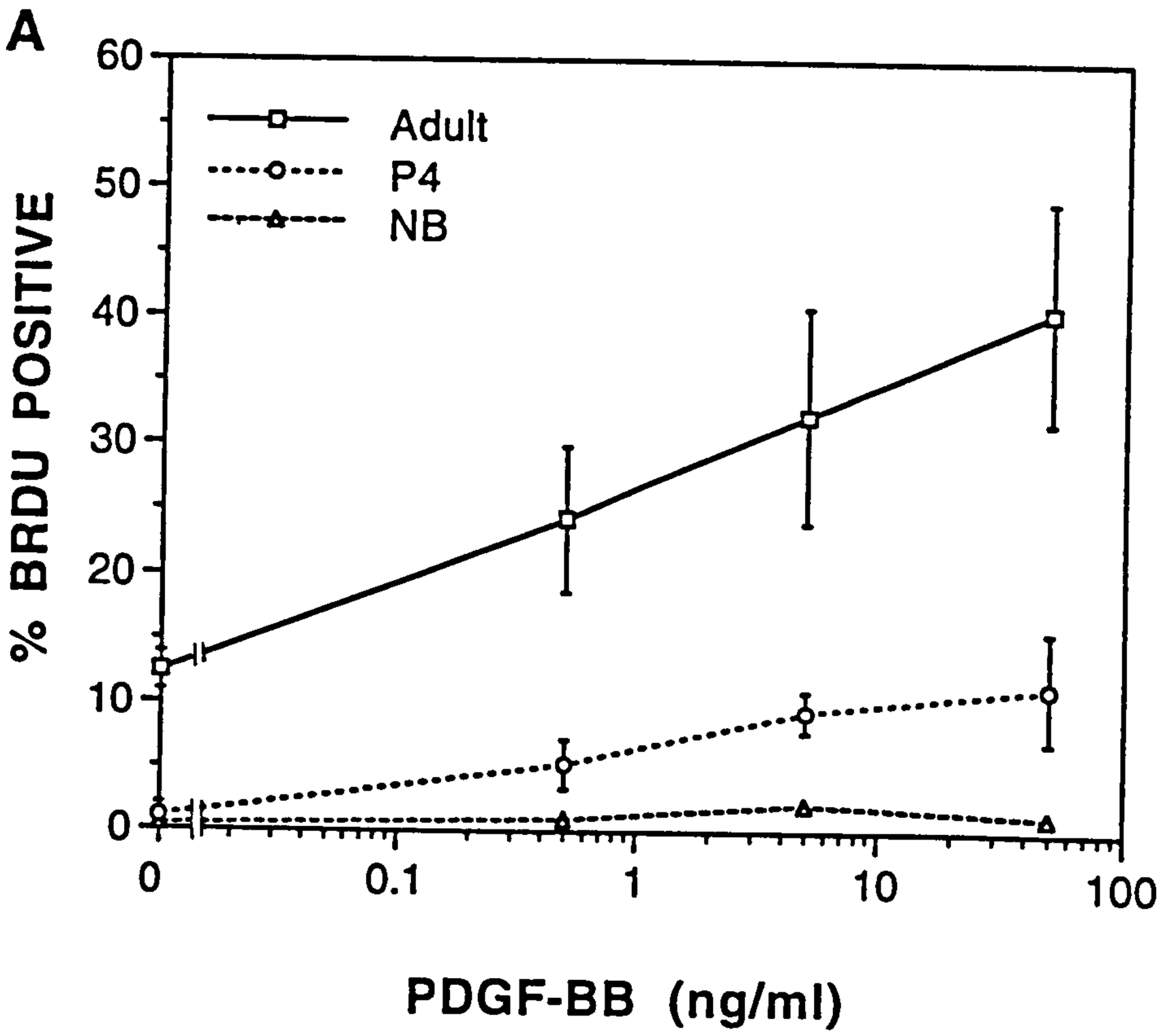

B
30
25
20
15
10
5
0
% BRDU POSITIVE
Adult
P4
NB
0
0.1
1
10
100
FORSKOLIN (μM)

Figure 5.10 Mitogenic potential of PDGF-BB plus forskolin in Schwann cells pre-cultured in serum-containing medium for 5 days.

Schwann cells from newborn, postnatal day 4 (P4) and adult sciatic nerves were cultured in serum-containing medium for 5 days, then the purified Schwann cells were exposed to (A) various concentrations of PDGF-BB in constant forskolin (2μM), (B) various concentrations of forskolin in constant PDGF-BB (50ng/ml) for 44 hr. BrdU was used in the last 20 hr of the assay. The results show that PGDF-BB plus forskolin are hardly mitogenic for newborn Schwann cells and show age-dependent mitogenic activity for P4 and adult Schwann cells.


A
60
50
40
30
20
10
0
% BRDU POSITIVE
Adult
P4
NB
0
0.1
1
10
100
PDGF-BB (ng/ml)

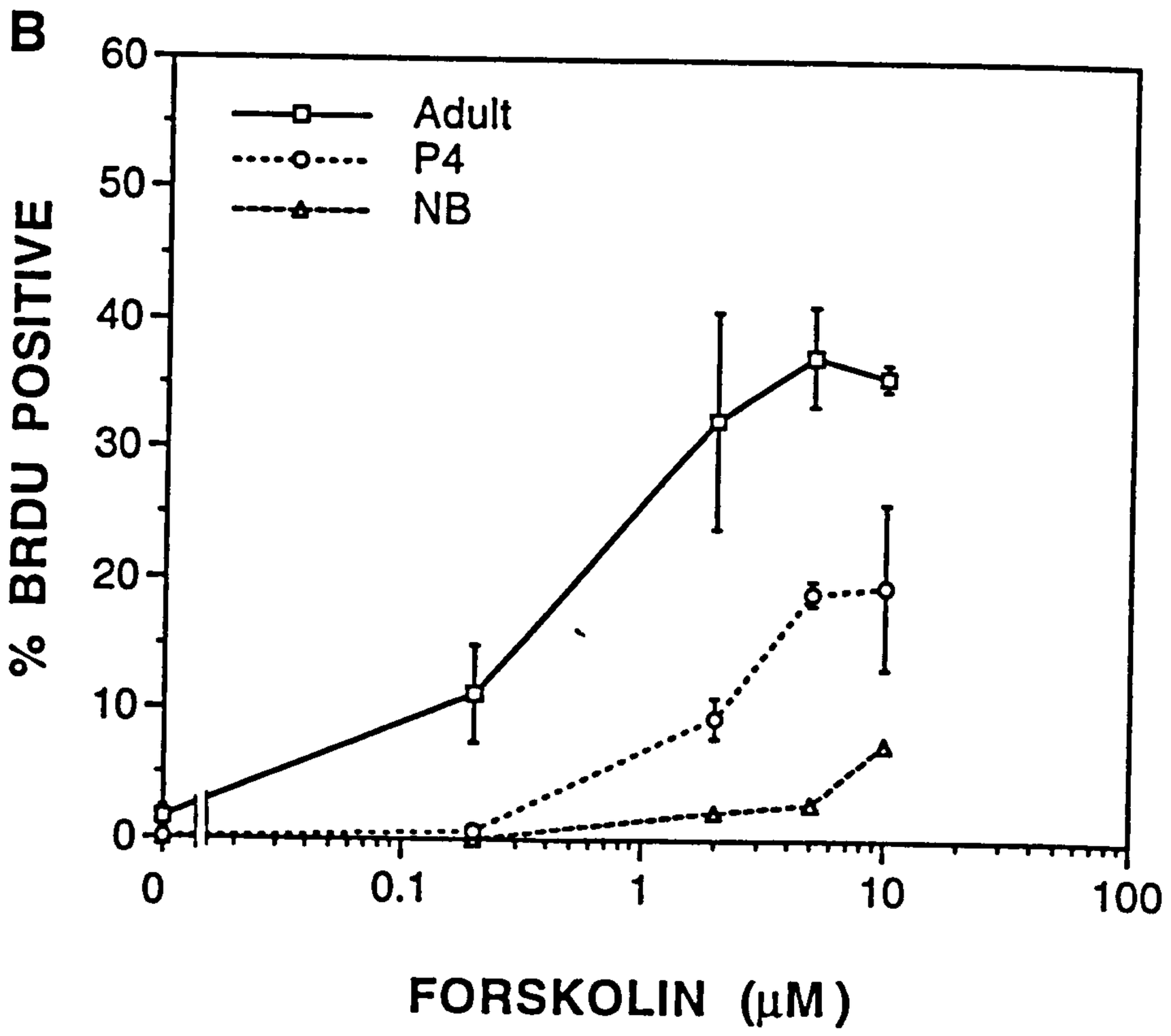
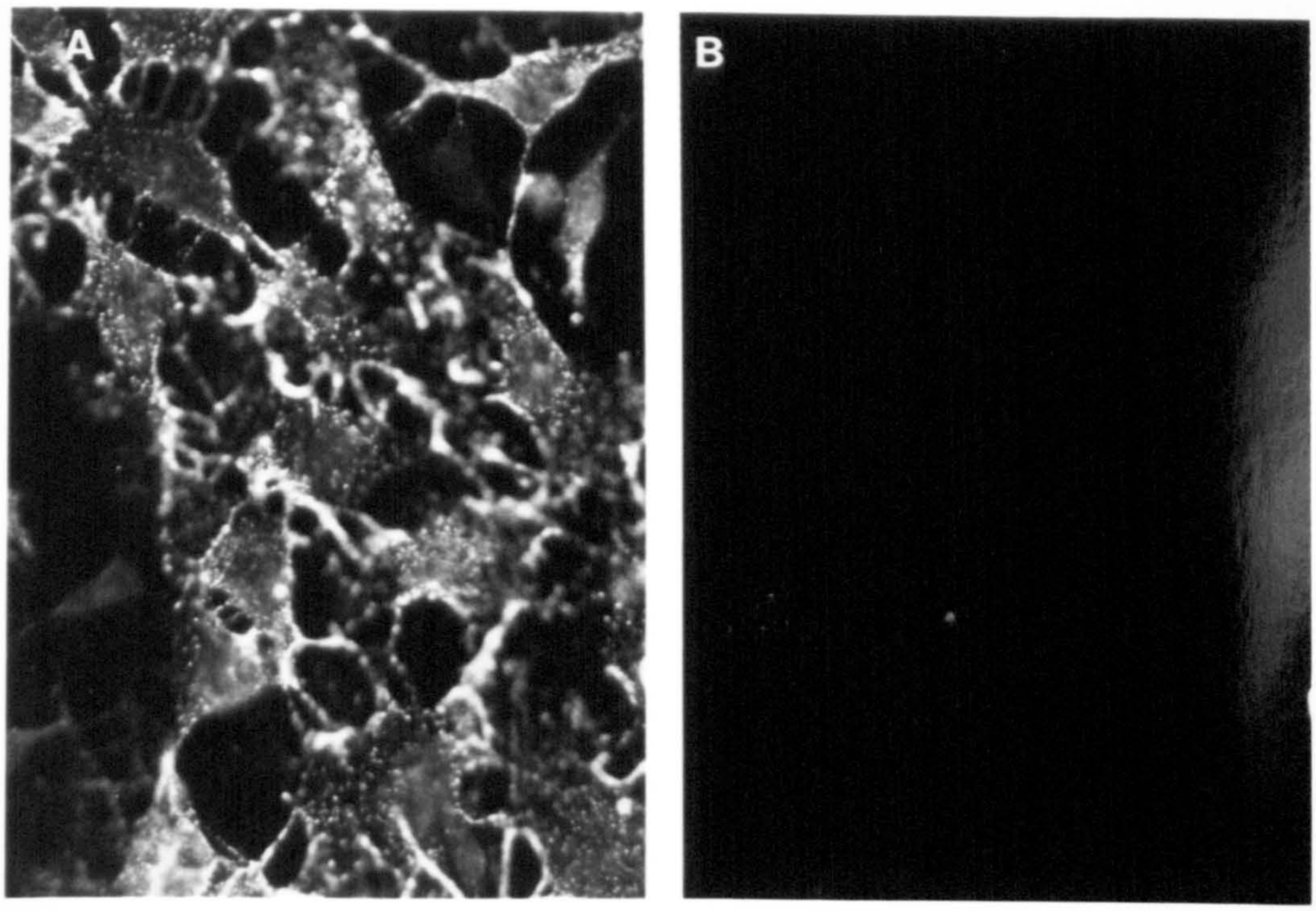

B
60
50
40
30
20
10
0
% BRDU POSITIVE
Adult
P4
NB
0
0.1
1
10
100
FORSKOLIN (μM)

Figure 5.11 TGFβ stimulates DNA synthesis of precursors in the presence of the non mitogenic combination of FGF-2 plus forskolin.

Schwann cell precursors dissociated from E14 rat nerves were immediately cultured in FIF medium (FGF-2 plus IGF and Forskolin) (A,B) or FIF medium plus TGFβ-1 (C,D) for 20 hr. A 1.5 hr BrdU pulse was used at the 18.5 hr point. The precursors were double-labelled with L1 antibodies (A,C) and BrdU antibodies (B,D). Magnification 700X


A
B

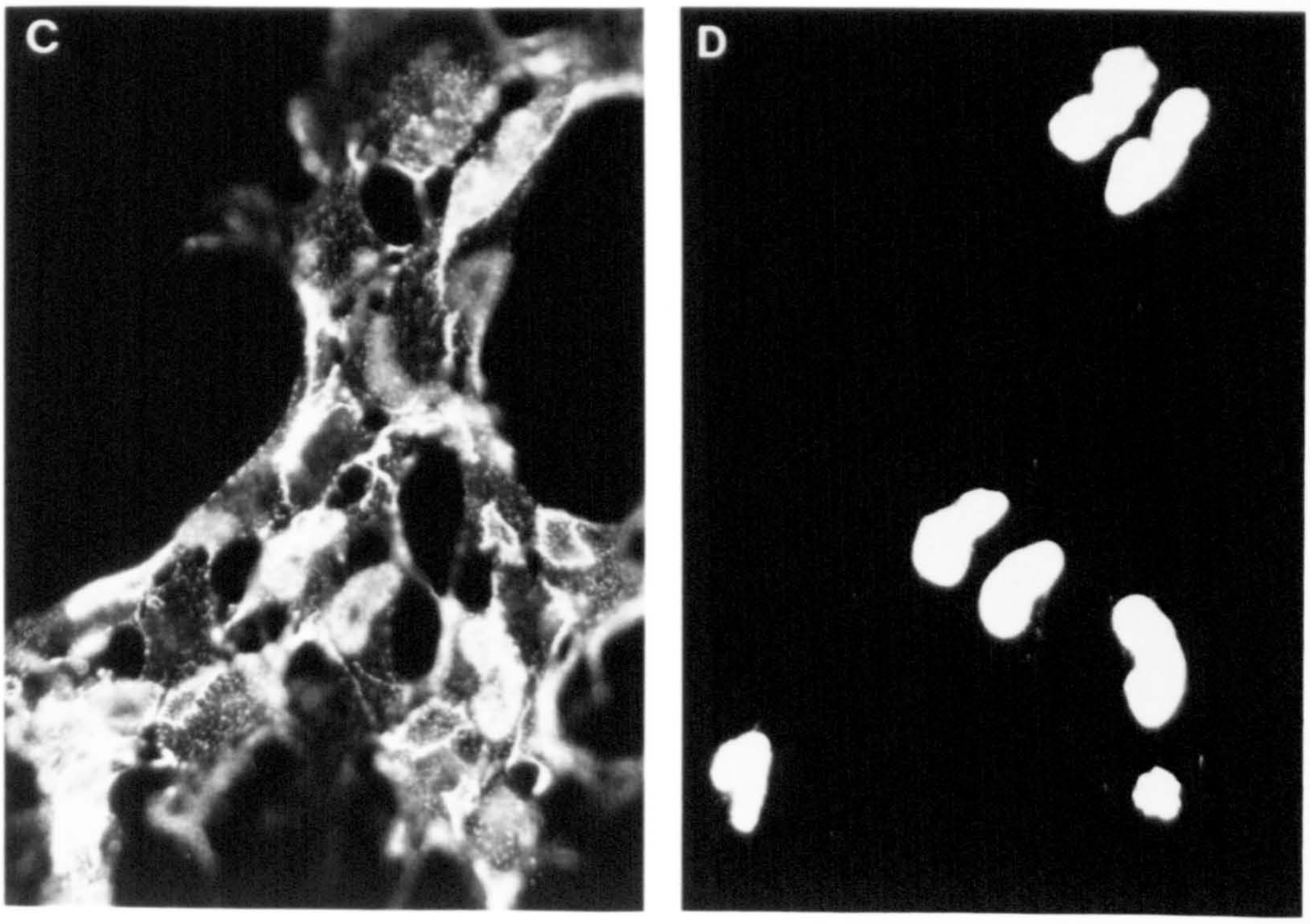
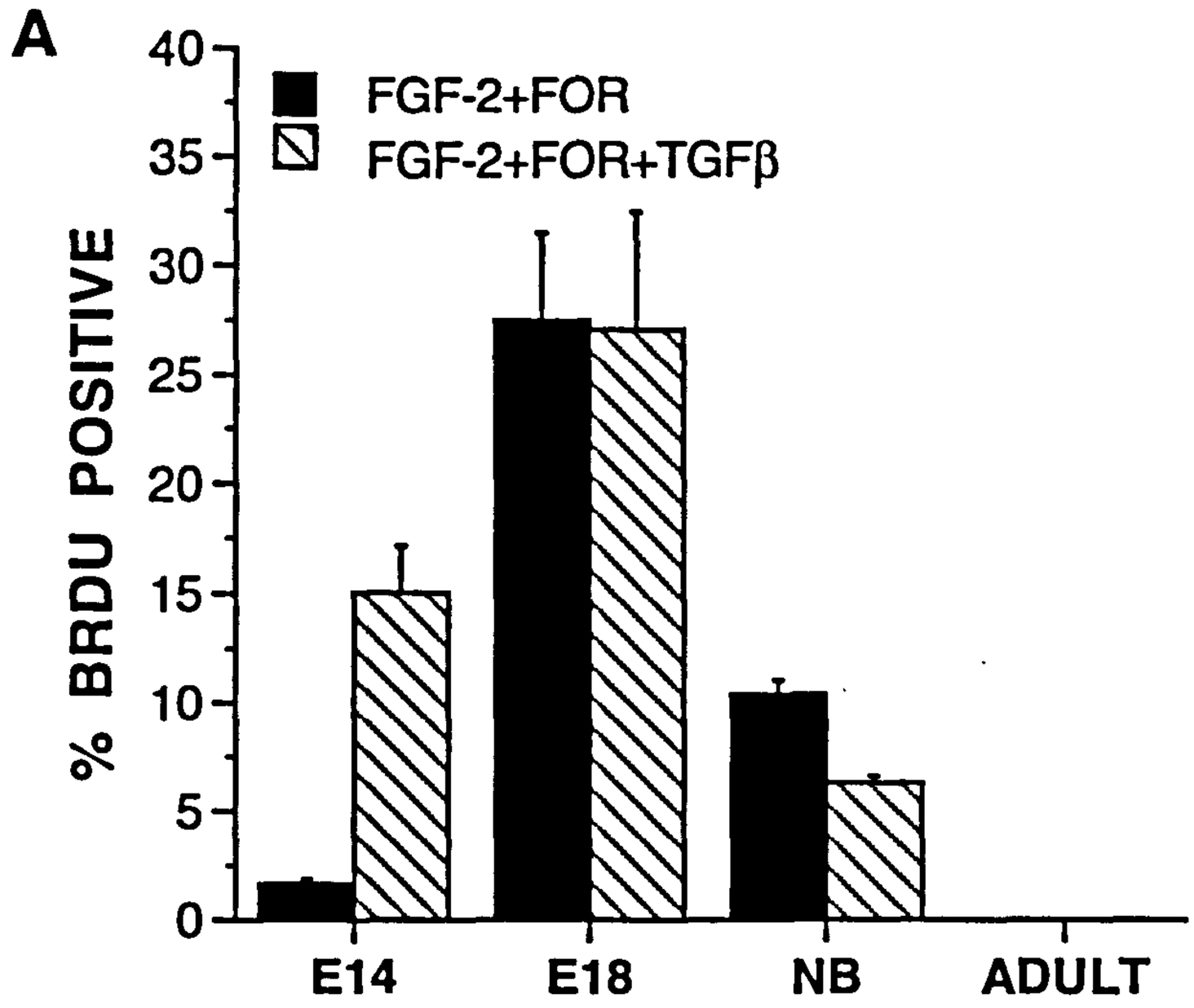

C
D

Figure 5.12 Mitogenic regulation of TGFβ in cells of the Schwann cell lineage

(A) Cells dissociated from E14, E18, newborn and adult sciatic nerves were immediately cultured in defined medium containing FGF-2 (180pM) plus forskolin (5μM) or FGF-2 (180pM) plus forskolin (5μM) and TGFβ1 (1ng/ml) for 20 hr. BrdU was used in the last 1.5 hr of the assay. (B) Schwann cells from newborn, 4 day and adult sciatic nerves were pre-cultured in serum-containing medium for 5 days, and then exposed to FGF-2 (180pM) plus forskolin (5μM) or FGF-2 (180pM) plus forskolin (5μM) and TGFβ1 (1ng/ml) for 44 hr, BrdU was used for the last 20 hr of the assay. The results show that TGFβ produces both a positive and negative effect on DNA synthesis in these cells


A
FGF-2+FOR
FGF-2+FOR+TGFβ
% BRDU POSITIVE
0
5
10
15
20
25
30
35
40
E14
E18
NB
ADULT

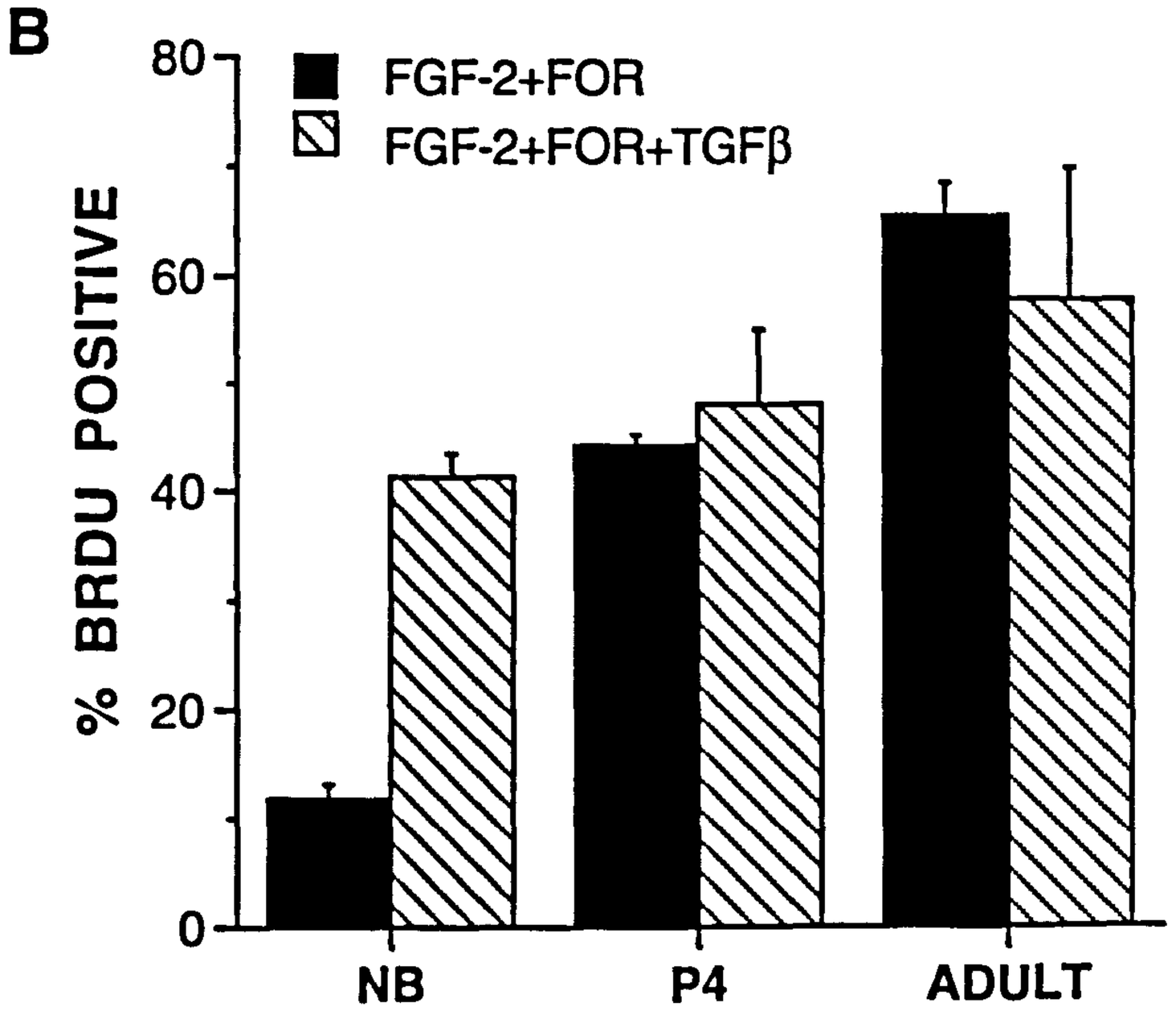

B
FGF-2+FOR
FGF-2+FOR+TGFβ
% BRDU POSITIVE
0
20
40
60
80
NB
P4
ADULT

Figure 5.13 TGFβ inhibits DNA synthesis of Schwann cell precursors induced by NDFβ.

E14 Schwann cell precursors freshly dissociated from nerve were cultured in defined medium either containing NDFβ-2 (A,B) or TGFβ plus NDFβ-2 (C,D) for 20 hr. A 1.5 hr BrdU pulse was used at the 18.5 hr point. The precursors were double-labelled with L1 antibody (A,C) and BrdU antibody (B,D). Magnification 70X

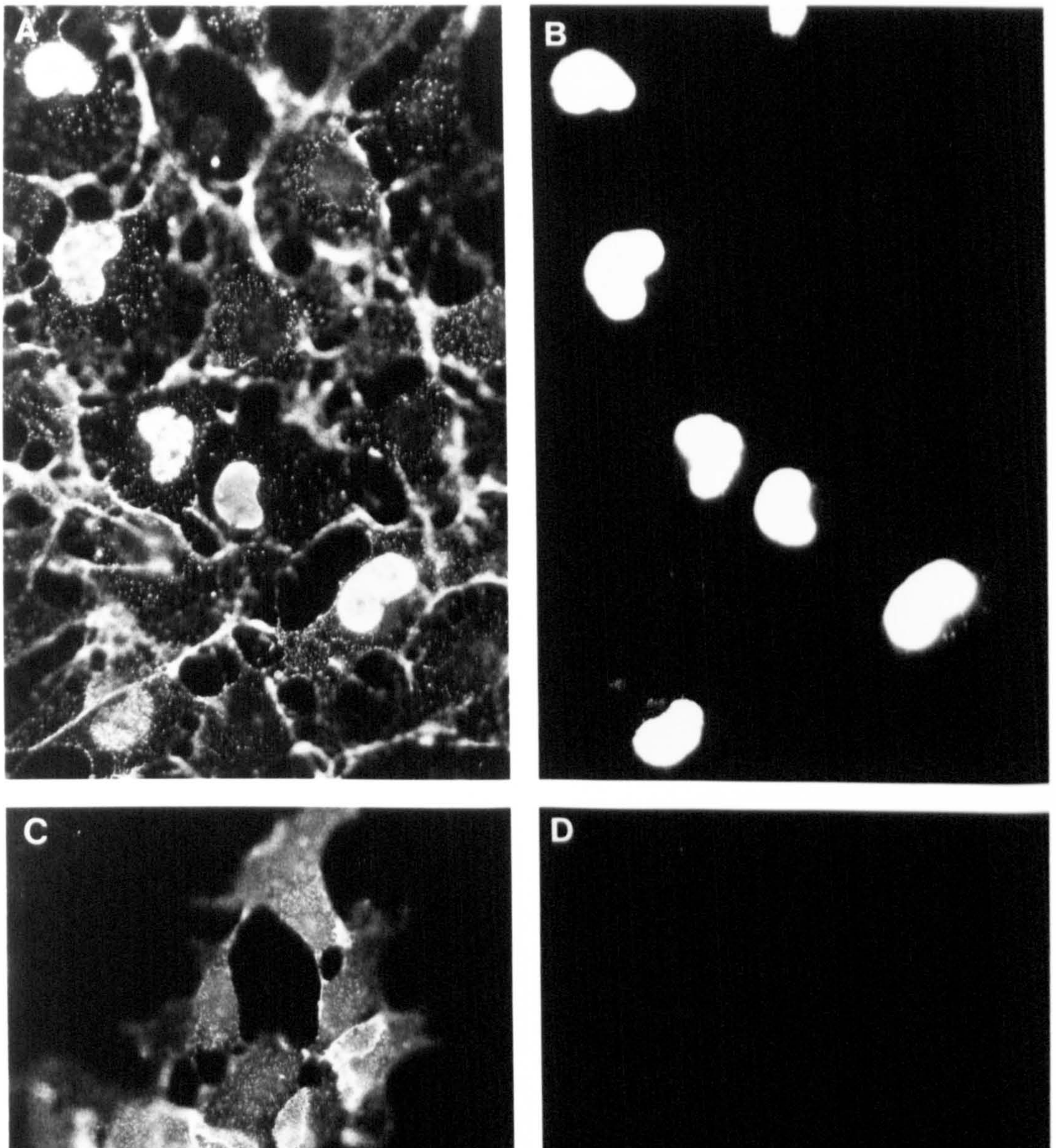

A
B
C
D

Figure 5.14 TGFβ suppresses DNA synthesis in precursors stimulated by NDFβ

Schwann cell precursors dissociated from E14 sciatic nerve were immediately cultured in defined medium containing NDFβ-2 (400pM) or NDFβ-2 (400pM) plus TGFβ1 (1ng/ml) for 20 hr. A 1.5 hr BrdU pulse was used at the 18.5 hr point. The precursors were double labelled with L1 and BrdU antibodies. The result shows that TGFβ on top of NDFβ suppresses DNA synthesis in Schwann cell precursors.

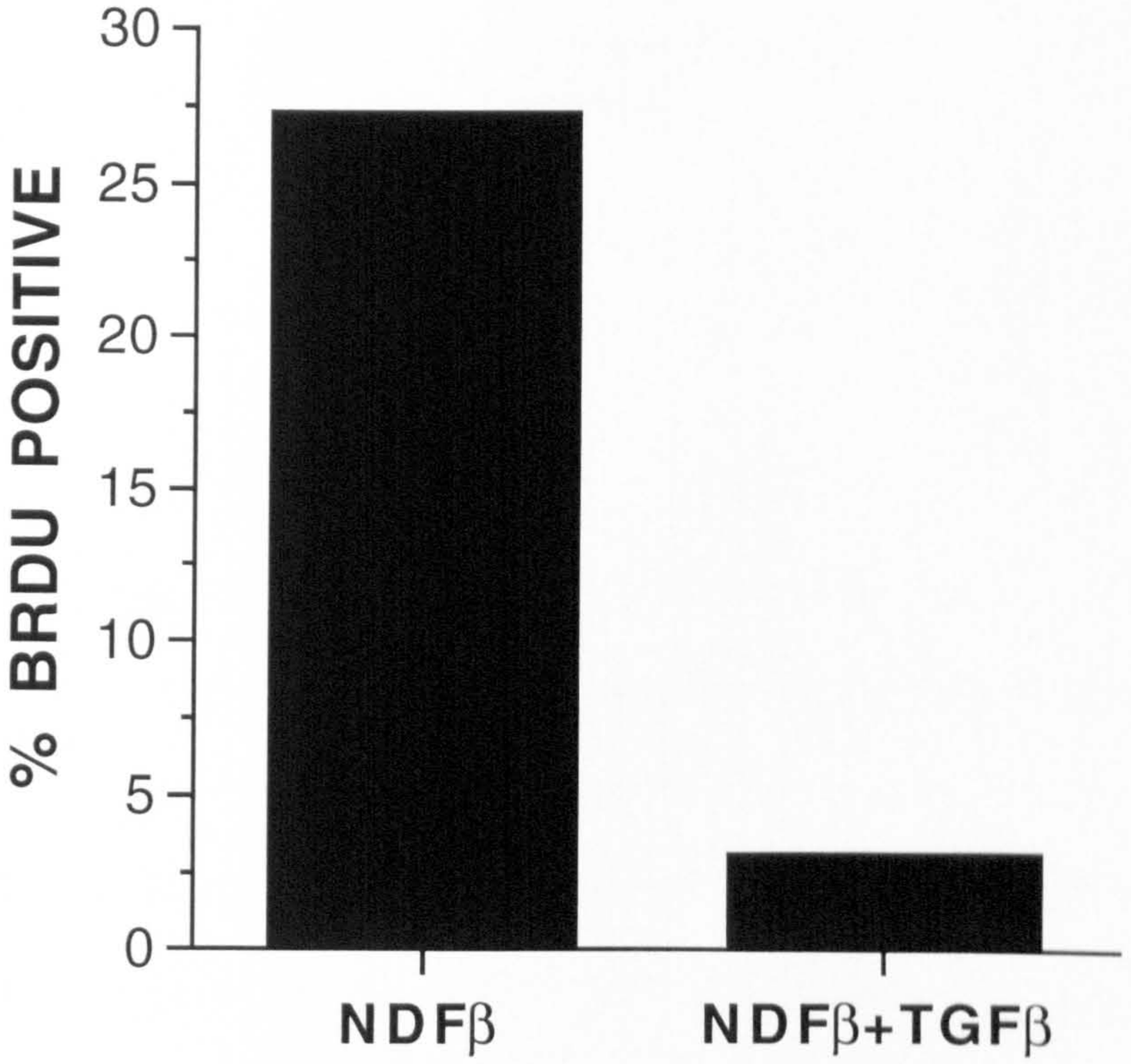

% BRDU POSITIVE
30
25
20
15
10
5
0
NDFβ
NDFβ+TGFβ

Figure 5.15 The effect of serum exposure on the mitogenic response of Schwann cells to FGF-2 plus forskolin

Schwann cells dissociated from newborn or adult sciatic nerves were either exposed to FGF-2 (180pM) plus forskolin (5μM) immediately for 44 hr or cultured in serum-containing medium for 5 days then exposed to FGF-2 (180pM) plus forskolin (5mM) for 44 hr. BrdU was used for the last 20 hr of the assay in both conditions. The results show that the DNA synthesis is decreased in newborn Schwann cells and significantly increased in adult Schwann cells after serum exposure.

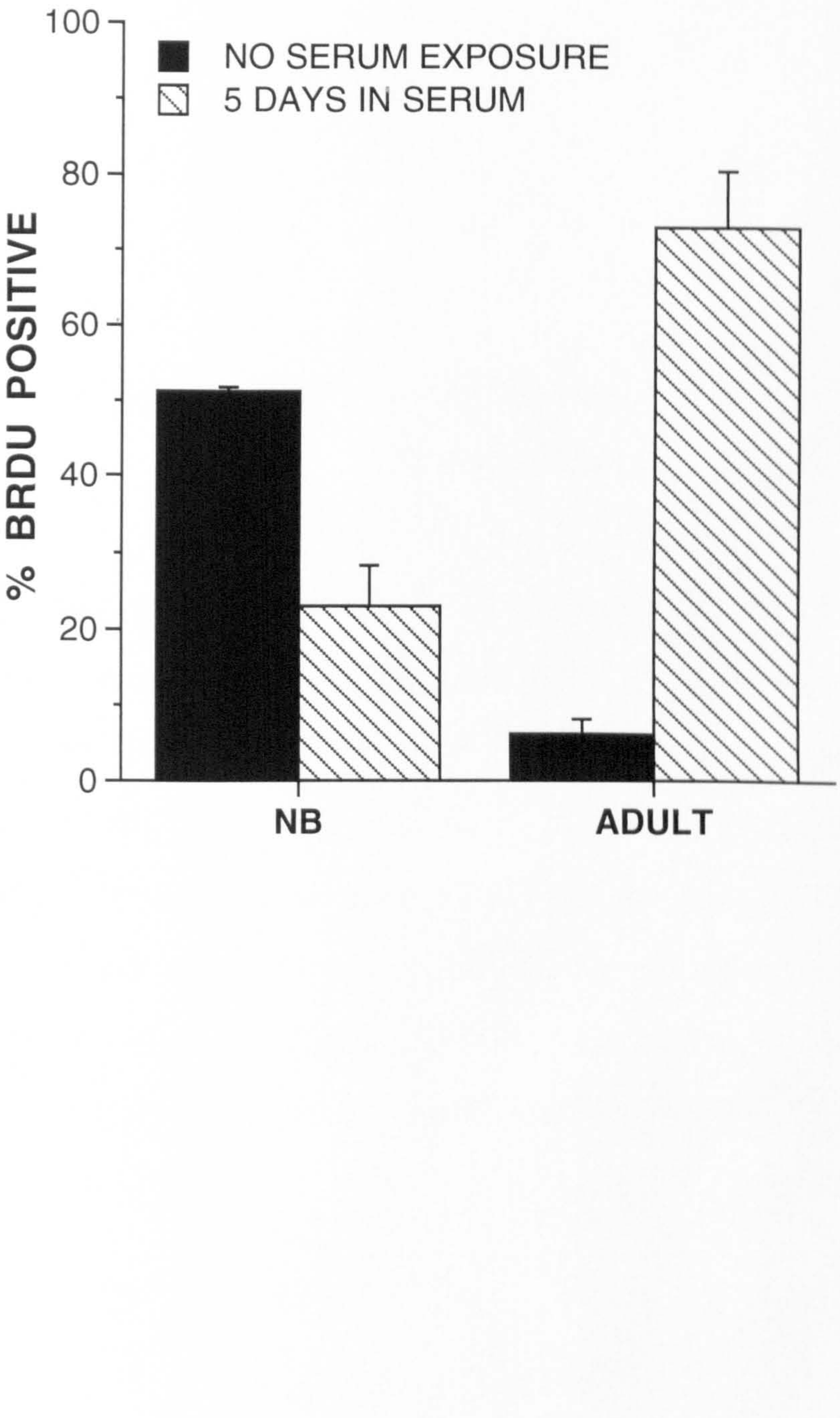
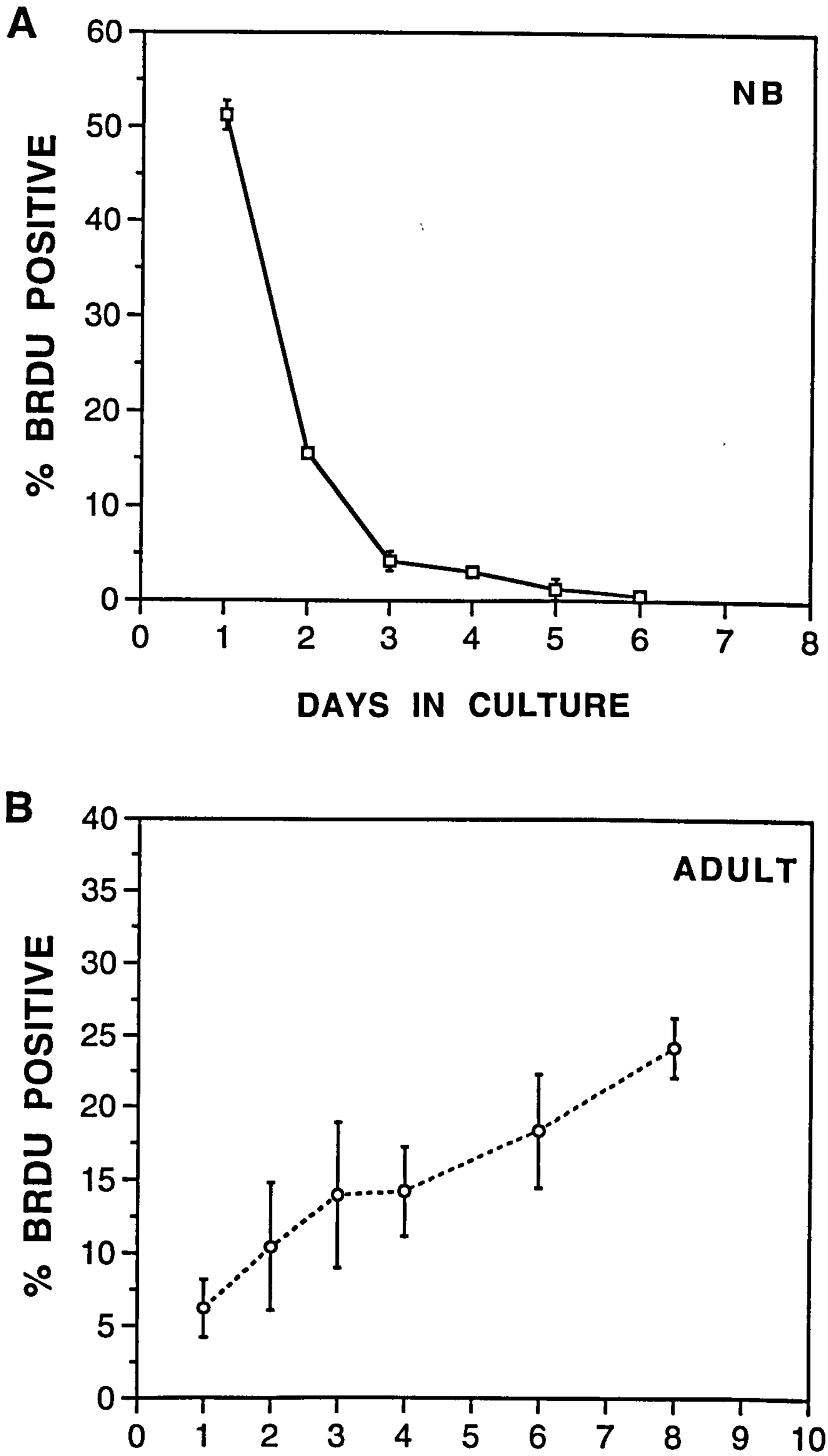

NO SERUM EXPOSURE
5 DAYS IN SERUM
% BRDU POSITIVE
100
80
60
40
20
0
NB
ADULT

Figure 5.16 The effect of culture period on the mitogenic response of Schwann cells to FGF-2 plus forskolin

Schwann cells dissociated from (A) newborn or (B) adult sciatic nerves were cultured in serum-free defined medium for 0-8 days. The cultured Schwann cells were then changed to FGF-2 (180pM) plus forskolin (5μM) containing medium at day 0, 1, 2, 3, 4, 5, 6, 8. Schwann cells were cultured in FGF-2 plus forskolin containing medium for 44 hr and BrdU was introduced to the culture for the last 20 hr of the assay. The results show that the mitogenic response of Schwann cells to FGF-2 plus forskolin is reduced dramatically in newborn animals (A) and gradually increases in adult animals (B).


A
NB
% BRDU POSITIVE
DAYS IN CULTURE
B
ADULT
% BRDU POSITIVE
DAYS IN CULTURE

CHAPTER 6

GENERAL DISCUSSION

This study has systematically investigated the survival, proliferation, maturation and differentiation of cells in the Schwann cell lineage and focused on the molecular interactions between neurons and Schwann cell precursors\Schwann cells. The thesis aimed: firstly, to identify the neuron-derived survival factors for Schwann cell precursors; secondly, to investigate the interactions between Schwann cell precursors and neurons that led to survival, proliferation, maturation and differentiation of Schwann cell precursors; thirdly, to explore the mitogenic potential of NDF, FGF, TGFβ and PDGF-BB throughout Schwann cell precursor and Schwann cell development including an analysis of the mitogenic response to these growth factors in cultured adult Schwann cells. This study also introduced a method of obtaining purified DRG neurons, and demonstrated that DNA synthesis assays performed at different stages of Schwann cell culture gave very different results. These results may relate to different proliferation events that occur during normal development and Wallerian degeneration.

The identification of the neuron-derived survival signal is a important step in understanding the development of the PNS. The study present here strongly supports the idea addressed previously (Jessen et al., 1994) that the interaction of neurons and Schwann cell precursors supports Schwann cell precursor survival. The survival signals were found to be the molecules located in both neuron conditioned medium and on or associated with neuronal surface. This thesis also provides strong evidence (see below) that this signal is β forms of the NDF family.

Selecting NDF as a candidate for neuronal survival signal was based on the fact that this newly purified and sequenced growth factor belongs to a novel growth factor family, [NDF, heregulin(HRG), glia growth factor(GGF) and ARIA]. Some members of this family (pure and impure GGF) have been shown to play a important role in Schwann cell proliferation, and have a more potent mitogenic activity in comparison to other Schwann cell mitogens (Stewart et al., 1992; 1995). Meanwhile, this growth factor has also been shown to be highly expressed in the embryonic peripheral nervous system (Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994). Immuno-labelling and Western blotting observations in this study revealed that NDF was

strongly expressed on neurons but was not detectable in Schwann cell precursors. Interestingly, Schwann cells from the neonatal rat also appeared to express a weak NDF signal. Together, it seems likely that this molecule may act as a survival molecule for Schwann cell precursors. In this regard, NDF was tested in survival assays of these cells. As expected, NDF showed a marked potential to support Schwann cell precursor survival both in short-term and long-term culture, closely mimicking the survival signal from NCM (Jessen et al., 1994). Based on these observations, blocking experiments were designed by using soluble ErbB4 protein, since similar blocking experiments have been widely used to explore the function of identified molecules in other systems (Kalebic et al., 1994; Barres et al., 1993). The results in this study showed that soluble ErbB4 protein significantly reduced the survival activity both in NCM and on the neuronal surface. Taken together, all the observations presented here strongly support the idea that NDF acts as the key neuronal survival factor for Schwann cell precursors.

Previous reports from our group indicated that FGF could also support precursor survival in the presence of IGF (Jessen et al., 1994; Gavrilovic et al., 1995). The present study further explored the role of this molecule in the interaction of neurons and Schwann cell precursors. The results revealed that FGF-2 may also be involved in neuronally mediated precursor survival, but the survival contribution of this molecule in the neuronal survival signals for Schwann cell precursors appears to be minor. In the CNS, oligdendrocytes and their progenitors rely on multiple survival signals which are derived from neurons (Barres et al, 1992; 1994). The Schwann cell precursor may also rely on multiple neuronal signals for survival such as NDF, FGF and other unidentified factors, but it is most likely that NDF plays the key role in Schwann cell precursor survival during peripheral nerve development.

In vivo, Schwann cell precursors not only survive but also show vigorous proliferation (Stewart et al., 1993). Interestingly, NCM which supports Schwann cell precursor survival, was unable to stimulate DNA synthesis in these cells (Jessen et al., 1994). Does the proliferative signal for precursors derive from neurons? The experiment using very pure and immunolabelled neurons, showed that direct contact

with neurons or axons promotes DNA synthesis in the Schwann cell precursor. The difference between NCM and direct contact with neurons in terms of mitogenic potential may relate to the differing concentration of mitogens in these two different conditions. With regard to identifying the neuronal mitogenic signal for precursors, this study revealed that NDF alone and TGFβ in the presence of FGF plus forskolin stimulated DNA synthesis in Schwann cell precursors. In view of the recent identification of NDF as a neuronal associated mitogen for Schwann cells (Morrissery et al., 1995), it is also possible that NDF acts as an axon-associated mitogenic signal for Schwann cell precursors. Although this study also showed that TGFβ can stimulate DNA synthesis in Schwann cell precursors in the presence of other factors, some other observations in this study indicated that TGFβ can either promote or suppress DNA synthesis in both Schwann cell precursors and Schwann cells, indicating that this molecule may act in a more complicated way to control the proliferation of these cells. Recent studies on TGFβ receptors suggest that different combinations of TGFβ receptors might be involved in triggering different functional pathways (Waran et al 1992; Massague et al 1994). Therefore, exploring TGFβ receptor expression and functional analysis of the receptor combination in the Schwann cell lineage will be one of the approaches for future study.

Schwann cell precursors proliferate and develop into Schwann cells in vivo. The conversion of the Schwann cell precursor to Schwann cell has been defined as appearing between E15-E17 in the rat by showing an alteration of antigenic phenotype, survival ability and mitogenic response potential (Jessen et al., 1994). This study revealed that direct contact with neurons led to maturation of Schwann cell precursors, as does NCM (Jessen et al., 1994). Therefore, it is clear that neuronal molecules play an important role during this conversion. NDF has been shown to support this conversion on schedule (Dong et al., 1995). It seems likely that NDF is the neuronal molecule that promotes the conversion of Schwann cell precursors to Schwann cells. Alternatively, it is also possible that NDF may only block the apoptosis of precursors without providing a positive signal for lineage progression.

During Schwann cell generation, Schwann cell precursors\Schwann cells also undergo vigorous proliferation. The molecular basis for this proliferation appears to change markedly as the cells mature. My studies show that FGF plus forskolin gradually becomes a mitogen as Schwann cell precursors convert to Schwann cells in vivo. In contrast, the mitogenic potential of TGFβ is reduced and in older rat Schwann cells, TGFβ slightly suppressed mitogenic activity triggered by FGF plus forskolin at later stages. NDF is a mitogen for Schwann cell precursors, and continually acts as a strong mitogen in Schwann cells. Interestingly, PDGF-BB plus forskolin, a well known Schwann cell mitogen in short term culture systems, may not act as mitogen for the Schwann cell during normal peripheral nerve development since this study shows that this combination does not stimulate DNA synthesis of freshly dissociated Schwann cells at any developmental stage. It is unclear why the proliferation rate of Schwann cells in vivo is markedly increased as Schwann cell precursors convert to Schwann cells (Stewart et al., 1993). One possibility is that with the increasing mitogenic potential of FGF-2, NDF and other unknown mitogens, the combination of these factors may drive Schwann cells towards massive proliferation, reaching a peak at E19-E20.

With the onset of myelination, Schwann cells recede from proliferation and undergo differentiation. Experiments in this study showed that Schwann cell precursors in contact with neurons can be induced to express relatively high levels of the myelin-related protein Po. Together with previous reports, in which elevated Po levels were also found in neonatal Schwann cells when these cells were co-cultured with neurons (Morrison et al., 1991; Brunden et al., 1990; 1992), this shows that neuronal molecules can promote Schwann cell differentiation. In addition, it is also known that there are soluble myelinogenic molecules in medium from cultured neurons since blocking direct contact between neurons and Schwann cells in a co-culture also results in induction of both Po and SCIP gene expression in Schwann cells (Bolin and Shooter, 1993). Although the evidence is clear that neuronal signals regulate the differentiation of Schwann cells the molecular nature of the neuronal differentiation signals are still generally unknown.

As mentioned above, parallel to Schwann cell differentiation is the rapid recession from proliferation of these cells. It is unknown what causes this recession. The experiments presented in this study, indicated that after birth, Schwann cells which were dissociated from the nerve and immediately exposed to potential mitogens, showed a significant decline in response to these mitogens compared with cells from embryonic animals. In the adult, freshly dissociated Schwann cells do not respond to any growth factors or their combinations when tested immediately after removal from the animal. One possibility for this mitogenic shift may relate to their corresponding receptor regulation since it has been shown that ErbB2 (neu), FGF receptor and PDGF receptors were downregulated in peripheral nerves in adult animals (Eccleston et al., 1992; Reddy and Pleasure, 1992; Cohen et al., 1992; Jin et al., 1993; Davis, 1993). Alternatively, it is also possible that the Schwann cell may respond to different mitogens after birth, but the parallel experiments in this study also demonstrated that after culturing in serum medium for 5 days adult cells show a significant mitogenic response to the same mitogens as cells from younger animals, making the requirement for different mitogens during postnatal development less likely.

Schwann cells in the adult animal are quiescent, but when adult nerve is transected Schwann cells in the nerve undergo de-differentiation and proliferation (Abercrombie and Johnson, 1946; Clemence et al., 1989; Komiyama and Suzuki, 1992). This proliferation event may relate to myelin debris, macrophage invasion and also axonal surface exposure (Yoshino et al., 1978; Baichwal and DeVries, 1989; Clemence et al., 1989). My experiments reveal that an alteration in the biological activity of adult Schwann cells may also contribute significantly to this proliferation since this thesis shows that adult Schwann cells become gradually more potent in response to mitogens if they are deprived of axonal contact in vitro. The molecular basis for this alteration is unclear. One of the most likely explanations for this is that the upregulation of corresponding growth factor receptors in these cells after peripheral nerve transection may lead to restoration of proliferation. With regard to Schwann cell mitogens during Wallerian degeneration, NDF, and FGF or PDGF-BB in the presence of forskolin are potent mitogens for Schwann cells, especially for adult Schwann cells. TGFβ in contrast, acts negatively on DNA synthesis triggered by FGF

plus forskolin, suggesting its function may involve suppression of proliferation which is in agreement with a previous report that TGFβ slightly inhibited Schwann cell proliferation triggered by neurons (Guenard et al., 1995).

I have also introduced a immunopanning method, in which more than 95% pure DRG neurons were obtained. Immunopanning methods have previously been used to purify oligodendrocytes, Schwann cells and retinal ganglion cells (Barres et al., 1992; Lindsey et al., 1994; Cheng et al., 1995). The two great advantages of this method are firstly, that the neurons are highly pure, secondly, the purification procedure takes only 2-3 hrs, and only one day of culture is required to obtain extensive neurite outgrowth. As we know from this study that Schwann cells cultured in serum for several days appear to significantly alter their biological activity, it is also possible that neurons in culture for several days or weeks may also change their molecular expression or molecular secretions. A study of FGF expression in cultured DRG neurons indicated that the FGF-2 and FGF-1 expression on neurons changed during 2 to 30 days in culture (Neuberger and DeVries, 1993). In this regard, neurons obtained by the present immunopanning method, may more closely mimic the natural neurons in vivo in terms of molecular expression, in comparison to most other methods in which neurons were cultured and exposed to serum for several days (Salzer et al., 1980), This should allow us to further explore the key molecules in the interaction between neurons and Schwann cells.

The second technical innovation introduced in this study is the comparison of two different mitogenic assays. The routine mitogenic assay for Schwann cells has always involved culturing Schwann cells in serum for several days. My experiments showed that Schwann cells purified using this procedure alter their biological activity and that this change shows similarities with the change that takes place during Wallerian degeneration in terms of mitogenic responsiveness. In contrast, to understand the mitogenic response of Schwann cells in normal nerve freshly dissociated Schwann cells are required.

Future studies

The molecular interactions between Schwann cells and neurons which lead to the differentiation of Schwann cells are still unclear. Some recent studies have indicated that TGFβ may play a important role in inhibiting proliferation of Schwann cells driven by neurons in a co-culture system (Guenard et al., 1995), upregulating L1 and N-CAM and downregulating the expression of myelin-related proteins including Gal-C, Po, MAG and MBP (Mews and Meyer, 1993; Chandross et al., 1995; Guenard et al., 1995; Einheber et al., 1995; Stewart et al., 1995). Thus TGFβ may act as a neuronal signal to drive Schwann cells into the non-myelin-forming Schwann cell pathway. As previously described, neurons from the SCG did not induce non-myelin-forming Schwann cells to express Po while neurons from the DRG did (Brunden et al., 1992). Therefore, it is possible that some unidentified molecules which are expressed by SCG, drive Schwann cells into the non-myelin-forming path. TGFβ may act as one of these molecules. Using the immunopanning method introduced in this thesis it should be possible to co-culture Schwann cells with purified neurons from different sources (SCG, DRG) or different ages (E14, newborn). TGFβ neutralizing antibody will be used to block the different neuronal signals from different sources (SCG, DRG) in these co-cultures, to determine whether TGFβ acts as a neuronal non-myelin signal.

The studies presented in this thesis also indicated that alteration of the biological activity of adult Schwann cells may contribute to the proliferation of these cells in both long term culture and during Wallerian degeneration. The molecular basis for this alteration may relate to receptor regulation in these cells. Future studies on receptor expression during development and also receptor regulation in different culture conditions might help us to understand variations in proliferation in Schwann cells during development and Wallerian degeneration. Based on the present study, NDF and FGF but not PDGF-BB are likely to be mitogens for Schwann cells during development. Therefore, one area of future studies will focus on the regulation of FGF-R and ErbB receptors during development by using immunocytochemistry, Western blotting and RNAse protection assays.

One other line of future research will focus on the developmental alteration of Schwann cell precursors in those mice with specific gene knock outs. Recent studies showed myelin disruption of Schwann cells in Krox-20 and Oct6/SCIP knock out mice (Herdegen et al., 1994; Bermingham et al., 1995). It is of interest to further study the role of these genes in Schwann cell development and myelination in vitro and co-culture system.

CHAPTER 7

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